ANTIBACTERIAL EFFECT OF VARIOUS CONCENTRATIONS OF SODIUM HYPOCHLORITE AND 2% CHLORHEXIDINE COMBINED WITH PROTEOLYTIC ENZYME AGAINST ENTEROCOCCUS FAECALIS BIOFILM – AN INVITRO STUDY

Dissertation submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment for the degree of

MASTER OF DENTAL SURGERY

BRANCH – IV

CONSERVATIVE DENTISTRY AND ENDODONTICS

2016- 2019

ENDORSEMENT BY THE H.O.D. PRINCIPAL / THE HEAD OF THE INSTITUTION

This is to certify that **Dr. S.ELANGOVAN**, Post Graduate student (2016–2019) in the Department o Conservative Dentistry and Endodontics, K.S.R. Institute of Dental Science and Research, has done th dissertation titled**"ANTIBACTERIAL EFFECT OF VARIOUS CONCENTRATIONS OF SODIU HYPOCHLORITE AND 2% CHLORHEXIDINE COMBINED WITH PROTEOLYTIC ENZYM AGAINST ENTEROCOCCUS FAECALIS BIOFILM – AN INVITRO STUDY"** under our guidan and supervision in partial fulfillment of the regulations laid down by **TheTamil Nadu Dr. M.G Medical University**, Chennai – 600 032 for **M.D.S.,** (Branch – IV) **CONSERVATIVE DENTIST AND ENDODONTICS** degree examination.

9/2/19

Seal and Signature of H.O.D.

Dr.Sebeena Mathew, M.D.S.,

Professor and Head

PROFESSOR & HOD,
DEPT OF CONSERVATIVE
DENTISTRY & ENDODONTICS.
K S R Institute of Dental
Science & Research,
K. S. R. KalviNagar,
Thiruchengode, Tamil Nadu.

Seal and signature of Principal

Dr. G. S.Kumar,M.D.S.,

Principal

PRINCIPAL,
K.S.R. INSTITUTE OF DENTAL
SCIENCE & RESEARCH,
K.S.R. KALVI NAGAR,
THOKKAVADI POST,
TIRUCHENGODE - 637 215

DECLARATION BY THE CANDIDATE

TITLE OF DISSERTATION	**ANTIBACTERIAL EFFECT OF VARIOUS CONCENTRATIONS OF SODIUM HYPOCHLORITE AND 2% CHLORHEXIDINE COMBINED WITH PROTEOLYTIC ENZYME AGAINST ENTEROCOCCUS FAECALIS BIOFILM – AN INVITRO STUDY**
PLACE OF STUDY	K.S.R Institute of Dental Science and Research
DURATION OF COURSE	3 Years (2016-2019)
NAME OF THE GUIDE	Dr.Boopathi. M.D.S.,
HEAD OF THE DEPARTMENT	Dr.Sebeena Mathew, M.D.S.,

I hereby declare that no part of the dissertation will be utilized for gaining financial assistance for research or other promotions without obtaining prior permission from the principal, K.S.R Institute of Dental Science and Research, Tiruchengode. In addition, I declare that no part of this work will be published either in print or electronic without the guide who has been actively involved in this dissertation. The author has the rights reserved for publishing the work solely with prior permission of the principal, K.S.R Institute of Dental Science and Research, Tiruchengode.

2/2/19

Head of the Department

Signature of candidate

CERTIFICATE BY THE GUIDE

This is to certify that the dissertation titled "ANTIBACTERIAL EFFECT OF VARIOUS CONCENTRATIONS OF SODIUM HYPOCHLORITE AND 2% CHLORHEXIDINE COMBINED WITH PROTEOLYTIC ENZYME AGAINST ENTEROCOCCUS FAECALIS BIOFILM – AN INVITRO STUDY" is a bonafide research work done by Dr. S. ELANGOVAN in partial fulfillment of the requirements for the degree of **MASTER OF DENTAL SURGERY** in the speciality of **CONSERVATIVE DENTISTRY AND ENDODONTICS**

09/02/2019

Date: 9/2/19

Place: **Tiruchengode**

Signature of the guide

Dr. Boopathi. T M.D.S.,

Professor

ACKNOWLEDGEMENT

First of all, I thank **GOD, THE ALMIGHTY**, for blessing me abundantly and for giving me the confidence and inclination to complete this Dissertation.

I express my sincere thanks to Chairman **Thiru. Lion. Dr.K.S.Rangasamy, MJF.,** Principal **Dr.G.S.Kumar, M.D.S**., K.S.R Institute of Dental Science and Research, Thiruchengode, for allowing me to pursue this course and avail the facilities of this college.

I express my deepest gratitude to my Professor and Head of the Department **Dr.Sebeena Mathew, M.D.S.,** for her expert and never-failing guidance, valuable suggestions, constant encouragement and support with kindness in all aspects of my career.

With overwhelming gratitude I thank my Professor **Dr.Boopathi.T, M.D.S.,** my guide and mentor, who has taken extreme pain and patience in helping me throughout with her immense support and valuable guidance to complete my dissertation successfully within the stipulated period.

I am extremely thankful to **Dr.K.Karthick , M.D.S., Dr Deepa N. T M.D.S** for their caring advices which has driven me to work with confidence in both academic and clinical studies.

I would like to thank my biggest source of strength, my parents,

Mr.G.Sekar,Mrs S Vijayaand my wife **Dr Hemalatha** whose unwavering, unselfish love, their expeditious encouragement and prayers have always been a pillar of support for me.

I would like to thank my super seniors **Dr.Loganathan, Dr.Poojithaviswanath, Dr.Abitha** for their clinical guidance and tips.

I would like to thank **Dr Prasanna M.D Microbiology department** Ramakrishna hospital who helped me to complete my thesis. I am also thankful to **Dr Muthu Kumar PhD Bharathiyar university, Tiruchy** for his ideas and guidance during Confocal laser scanning microscopy imaging. I thank the non-teaching faculty from the Department of Conservative Dentistry and Endodontics for their prompt and patient help throughout the course.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF GRAPHS

Introduction

The primary requiste for successful endodontic treatment is the complete debridement of root canal space i.e.,removal of all vital and nonvital tissues, removal of pathogenic microbes and its by-products from the root canal dentin.[1] The main concern about cleaning of root canal space is complete eradication of enterococcus fecalis which is considered to be one of the most important microorganisms involved in persistent periapical infections after root canal therapy.[2]

Enterococcus faecalis is a gram positive, nonspore forming, fermentative, facultative anaerobe. It is usually present in 24%-74% of persistent endodontic infections. It has the potential to survive in most tough environments i.e.,resistance to various antimicrobial agents such as detergents, glycerol, bile salts, heavy metals,ethanol,azides, dessication and extreme temperature conditions (10 ^{o} C to 45 ^{o} C).It has ability to survive at 60 ^{o} C for 30 mins and in extreme alkaline pH and salt concentrations also.[3,4,5]

Apart from its inherent capacity to survive under extreme environmental conditions as a single microorganism, it forms biofilms which has a resistance factor of about 1000 times more than the isolated single microorganism to antimicrobials, phagocytosis and antibodies.[6]

Biofilm consists of both organic and inorganic components such as carbohydrates, proteins, nucleic acids, salts , calcium, potassium, magnesium and Fe3+ ions. These components make 85% of biofilm volume. The remaining 15% is constituted by microbial cells.[7]

The resistant nature of the biofilm to various antimicrobial agents (antimicrobial tolerance) can be explained in four mechanisms[8]They are:

1.Extracellular polysaccharide matrix(provides physical barrier to antimicrobial agents).

2.Slow growth (nature of growth of bacterial cells in biofilm is slow when compared to bacteria present in a planktonic state. The planktonic organisms have a very slow growth phase when compared to the microorganisms in the biofilm. Subsequently, this results in slow intake of antimicrobial agents in to the bacterial cell)

3. Metabolic heterogeneity (Cells present in the deeper layers of biofilm experience different environmental conditions than those present in superficial layers. This promotes different metabolic characteristics among the microbial cells.

4.Presence of persisters (the persisters exist, they exist in a phenotypic state is very resistant to antimicrobial agents).[7]

Sodium hypochlorite is considered to be potent and is the gold standard of all irrigants used in endodontic therapy. Free chlorine is liberated when sodium hypochlorite is used as a root canal irrigant[9] and it has the property of dissolving organic structures present in the root canal. The tissue dissolving property of sodium hypochlorite is concentration dependent . A range of 0.5% to 5.25% concentration isused. Increasing the concentration of Naocl increases tissue dissolving property.[10]

The potent drawbacks of sodium hypochlorite such as tissue toxicity and adverse effects on dentin walls of the root canal are concenteration dependent.[11,12] Lower concentrations of sodium hypochlorite showed less subversive effects, but it showed decreased ability to penetrate the extracellular polysaccharide matrix of biofilm. Higher concenteration of sodium hypochlorite effectively dissolved the biofilm but it is with more adverse reactions in dentin.[13]

Chlorhexidene, an another potent antimicrobial agent is used as a root canal irrigant . Usually 2% concentrations of chlorhexidene is used. Chlorhexidene is less caustic than Naocl.[14] Despite being advantageous, Chlorhexidine has drawbacks such as inability to dissolve organic components, and it has reduced effectiveness on gram negative organisms.[14]

Trypsin ,a proteolytic enzyme meant for the degradation of proteins in to peptides disrupt the architectural pattern of proteins. It has been proved that proteolytic enzymes degrade the biofilm matrix showed synergistic effects with other antimicrobial agents.[15,16]

Thus, in the present study in order to maintain the desirable property of Naocl with minimal adverse reactions, trypsin, a proteolytic enzymes was used along with various concentrations of Naocl and chlorhexidene . These solutions were used on hydroxyapatite discs with enterococcus faecalis biofilm grown on it and viewed under confocal laser scanning microscopy for checking the viability of enterococcus faecalis.

AIM

To evaluate the antibacterial efficacy of various concentrations of sodium hypochlorite and Chlorhexidine combined with proteolytic enzyme in dentin - An invitro study.

OBJECTIVES

To evaluate the anti enterococcus faecalis efficacy using various concentrations of sodium hypochlorite (5.25%, 1% & 3%) and 2% Chlorhexidine combined with Trypsin, a proteolytic enzyme in root canal dentin.

Siquera et al investigated the antimicrobial efficiency of various root canal irrigants against black pigmented gram negative anaerobic rods and facultative anaerobes. The black-pigmented strains used were Porphyromonas endodontalis,Porphyromonas gingivalis,Prevotella intermedia, and Prevotella nigrescens. The facultative strains used were Enterococcus faecalis, Streptococcus mutans (clinical isolate), Streptococcus sanguis , and Streptococcus sobrinus . The root canal irrigants used were 0.5% NaOC1; 2.5% NaOC1; 4.0% NaOC1; 0.2% chlorhexidine digluconate; 2.0% chlorhexidine digluconate; 10% citric acid; and 17% EDTA. The agar diffusion test was performed . The results shown were 4% sodium hypochlorite showed larger areas of zones of inhibition than other irrigants used except 2.5% sodium hypochlorite. 2% chlorhexidene were better efficiency than citric acid and EDTA irrigants.[17]

Heling & N.P. Chandler conducted a study about various concentration of sodium hypochlorite (with and without EDTA), chlorhexidine and hydrogen peroxide used against Enterococcus faecalis biofilms on bovine incisors. Crowns of teeth removed and root length standardised into 4mm sections, then the specimens inoculated with Enterococcus faecalis strains and incubated for 5 days. Each specimens was irrigated with the following irrigation regime, 1. Saline for 10min(control); 2. 0.2% CHX for 10min; 3. H2O2 for 10min. 4 mixture of 0.1% CHX and 1.5% H2O2 for 10 min; 5 mixture of 1.8% CHX and 3% H2O2 for 10 min; 6 0.2% CHX for 5 min then 3% H2O2 for 5 min; 7 1% NaOCI for 10 min; 8 17% EDTA for 10 min; 9 3% HO2for 5 min then 1% NaOCl for 5 min; 10 1% NaOCI for 5 min then 3% H2O2 for 5 min; and 11 17% EDTA for 5 min then 1% NaOCl for 5 min. sterile round burs (ISO 023,025,027,029,031,033, and 035) were used for collecting the samples.Samples were inoculated in brain heart infusion broth at

37degree celsius for 24 hrs. They concluded that sodium hypochlorite and chlorhexidine had equal antibacterial effect. Synergistic effect of hydrogen peroxide on chlorhexidene seen at specific concentration.[18]

Spratt et al aimed to study the antimicrobial effect of four irrigants (2.25% Naocl, 0.2% chlorhexidine, 10% povidine iodine and 5 ppm colloidal silver) against single species biofilms (prevotella intermedia, preprosteptococcus micros, streptococcus intermedius, fusobacteriumnucleatum and enterococcus faecalis) which generated on cellulose nitrate membrane filters. After exposing the biofilm to the exposed irrigants, incubated for 15 to 60mins at 20ċ in fastidious agar plates. Colony forming units were counted to evaluate the antimicrobial effect. Results showed that Iodine andNaocl were effective against P.micros and P. Intermedia than chlorhexidine. At 15mins, no irrigants were effective against fusobacteriumnucleatum but all agents were effective after 1hr.[19]

Heling et al evaluated the minimal inhibitory concentration and minimal bacterial concentration of sodium hypochlorite(NaoCl) and sodium dichloroisocynurate(NaDCC) by protein determination method and also evaluated the cytotoxic effects of these two irrigants by using human fibroblast tissue culture. The tested microorganisms were Streptococcus sobrinus, Streptococcus salivarius, Enterococcus faecalis, and Streptococcus mutans. Results showed that the concentrations 0.02% NaDCC and 0.01% sodium hypochlorite had lethal effects on fibroblasts in tissue culture. At .01% concentration, Sodium hypochlorite was more toxic than NaDCC.[20]

L.M Sassone at al conducted a study using a contact test evaluating the antimicrobial efficiency of various concentrations of sodium hypochlorite (1% & 5%)and chlorhexidene.(0.12%, 0.5% & 1%). The samples of bacterial species obtained from American type culture collection (ATCC) were Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Porphyromonasgingivalis and Fusobacteriumnucleatum were submitted to a contact test. Irrigant Solutions were evaluated at different time intervals: immediately, 5 min, 15 min, and 30 min after contact and repeated 10 times. Results showed that 0.12% chlorhexidinewas failed eliminate the enterococcus at any time period but 1% & 5% sodium hypochlorite and 1% & 0.5% chlorhexidine eliminate microorganisms at all time periods. It was concluded that chlorhexidine concentrations with greater than 0.12% was needed for better antimicrobial activity.[21]

Oncag et al conducted a study about antibacterial properties and toxicity of 5.25% sodium hypochlorite(NaOCl), 2%chlorhexidine gluconate and 0.2% chlorhexidine gluconate plus 0.2% cetrimide. The Antibacterial effects and cytotoxicity of the above said irrigants were investigated on sixty freshly extractedsingle rooted teeth. Root length were standardised between 12 and 16 mm. Access preparation and instrumentation of canals were done 1 mm beyond the apical foramen with H files up to 50 size. The root ends of all the specimens sealed with epoxy resin and autoclaved at 121 degree Celsius for 15 mins for sterlization. Then the specimens were inoculated with enterococcus faecalis strain and irrigated with respective experimental solutions and sterile saline solution. They

concluded that chlorhexidene and cetrimide showed more antibacterial efficiency than sodium hypochlorite. Cetremide and 2%chlorhexidinegluconate were more effective, and exihibited more residual antibacterial effects and lower toxicity than 5.25% NaOCl solution.[22]

Ercan et al conducted an in vivo study on thirty root canals of single rooted teeth in 20 patients with necrotic teeth and periapical pathosis about the antibacterial activity of different root canal irrigants such as 2% chlorhexidine gluconate and 5.25% Sodium hypochlorite.Sampling were taken before and after root canal preparation using sterile paper points. Samples were incubated in trypticane soy agar for 5 – 7 days and colony forming units were counted. Both the root canal irrigantsshowed similar antibacterial effect.[23]

Sirtes at al aimed to study the effects of preheating of sodium hypochlorite on stability, pulp dissolving ability and antibacterial effect on human teeth.Totally 22 teeth (10 third molars and 12 premolars) extracted for orthodontic reasons were selected. Syringe heating device was used to heat the sodium hypochlorite solution. Pulp tissue were extracted from the teeth using spoon excavator and Five human pulp specimens were treated with the each of the irrigating solution. 1% NaOCl at 20°C,1%NaOClpreheatedto45°C,1%NaOClpreheatedto60°Candfinallya5.25%NaOCl solutionat20°C (negative control). Two pulp specimens were irrigated with 0.9% saline (positive controls). After treating with the irrigating solutions, pulp dissolution assay was performed using polyethylene mesh and filter paper. The remaining pulpal tissue were

weighed and compared with the pretreatedpulpal tissue weights. Chemical stability of sodium hypochlorite solution were evaluated at 45 and 60 degree . Chlorine present in the sodium hypochlorite solution were estimated after heating at different time intervals of 15,30 and 60mins. Estimation of chlorine was done by iodometric titration assay Antibacterial efficacy of sodium hypochlorite were studied after exposing sodium hypochlorite with the enterococcus faecalis biofilm.Test solutions were: NaOCl 0.001, 0.0001, and 0.00001% (wt/vol); control solutions were: 0.1 M sodium thiosulfate, 0.1 M sodium thiosulfate 0.001% NaOCl (1:10, vol/vol), and PBS. Solutions were either preheated to 45°C or cooled to 20°C in a water bath.The number of colony forming units were counted after inoculated in trypticase soy broth. Results shown were 1% /45 degrees celsiuswas as effective in pulp dissolving ability as 5.25%/20 degrees celsius. A 45 degree Celsius of heating of sodium hypochlorite showed better antibacterial effect than at 20degree celsius. The solution remain stable in all the temperaturestested.[24]

Berber et al conducted a study about effect of different concentrations of sodium hypochlorite(0.5%,2.5%and 5.25%) against Enterococcus faecalis . In this study, researchers used both rotary and hand instrumententation techniques. 180 extracted human teeth were inoculated with enterococcus faecalis for 21 days. Specimens were divided in to 12 groups depending on concentration of sodium hypochlorite and sterile saline solution. In each group the instrumentation techniques followed were hybrid technique, nickel titanium rotary 4mm short of apex, nickel titanium rotary instrumentation till the apex. After instrumentation, sampling of canals were done and colony forming units were counted. It was concluded that 5.25 % concentration of sodium hypochlorite showed effective antienterococcus faecalis activity than any other concentrations irrespective of technique used in this study.[25]

A. Ali Khademi et al compared the antibacterial substantivity of doxycycline, 2.6% sodium hypochlorie and 2% chlorhexidine gluconate. Eighty dentin tubes prepared from bovine incisors were infected and incubated with enterococcus faecalis for 14 days. The specimens were divided into five experimental groups as follows:- doxycycline Hcl, chlorhexidine gluconate, Naocl, Infected dentin tubes as positive control and sterile dentin tubes as negative control. Dentin chips were collected using round burs and cultured in using typticane soy broth, colony forming units were counted . Chlorhexidine showed significantly greater substantivity effect than sodium hypochlorite and doxycycline.[26]

Dunavant et al aimed to study the effect of 6% Naocl, 1% Naocl, smear clear, 2% chlorhexiine, REDTA and Biopure MTAD, against Enterococcus faecalis biofilm using a continuous flow system. In this study flow cell system was used for biofilm growth and submerged in tested irrigants for either 1 or 5 minutes. The killed bacteria was calculated as percentage for Naocl(>99.99%), 1%(99.78%), smear layer(78.06%), 2% chlorhexidine(60.49%), REDTA(26.99%) and Biopure MTAD (16.08%). Results showed that 1% Naocl and 6% Naocl were more efficient in removing enterococcus faecalis infection than the other testing solutions used.[27]

Olivieraet alconducted a study comparing the chlorhexidene gel and two different concentrations of sodium hypochlorite (1.5% &5.25%) about the antienterococcusfaecalis activity in root canal specimens. Eighty single rooted teeth were selected, decoronated and instrumented up to apical size 25 using hand files. Then specimens were irrigated with sodium hypochlorite and autoclaved for sterilizing the specimens. The specimens were

inoculated with enterococcus faecalis and incubated for 7 days. The specimens were divided into 5 groups according to the antimicrobial agents used in root canals. Groups were G1: (n=20)2% chlorhexidene gel . G2: (n=20) 1.5% sodium hypochlorite, G3: (n=20) 5.25% sodium hypochlorite, G4:(n=10) 10 teeth irrigated with distilled water ● G5: 10 teeth irrigated with natrosol gel. The sampling of root canal done in 3 says such as 1) before biomechanical preparation,2) post treatment (immediately after biomechanical preparation), 3)final(7days after biomechanical preparation). Colony forming units were counted.Results showed that 2% chlorhexidene gel and 5.25% sodium hypochlorite were efficient in removing enterococcus faecalis infection even after 7 days of preparation.[28]

Mercade et al conducted a study on different pH(12, 7.5& 6.5) of 4.2% sodium hypochlorite in enterococcus faecalis infected human root canals .One hundred sixty five root canals were prepared, inoculated and incubated with enterococcus faecalis for forty eight hrs. Specimens were divided into three groups : group 1:4.2% NaOCl pH 12; group 2:4.2% NaOCl pH 7.5; and group 3, 4.2% NaOCl pH 6.5. Samples from the root canals were collected, and bacterial growth was analyzed. None of the experimental solutions exhibited complete removal of infection. At pH 6.5, 4.2% sodium hypochlorite showed least bacterial growth compared to other experimental irrigants.[29]

M**aria et al** evaluated the minimal concentration of root canal irrigants required for the eradication of enterococcus faecalis biofilm. Dentin specimens were irrigated with sodium hypochlorite, chlorhexidene,citric acid, EDTA and phosphoric acid. Root canal irrigants applied for duration 1,5and 10 mins on enterococcus faecalis biofilm. The biofilm grew in the MPEC – HTP device at 37 degree Celsius for 24 hrs. This device has troughs with peg lid which was used to inoculate the enterococcus faecalis. Inoculated

device was placed on the rocking table for 24 hrs and incubated at 37degree celsius. Sodium hypochlorite showed better results when compared to chlorhexidene, citric acid, EDTA. Sodium hypochlorite was most effective irrigant at 1 min exposure against enterococcus faecalis biofilm at a concentration of .0065% whereas CHX showed antibibacterial activity at 2 % concentration and 5 mins of exposure against biofilm.[30]

Tirali et al aimed to study the effectiveness of various concentrations of sodium hypochlorite(0.5%, 2.5% and 5.25%) and octenisept (1%, 10%, 50%, 100%) against three standards microorganism stains such as staphylococcus aureus, E. Faecalis, Candida albicans. The microorganisms were exposed to disinfectants at different time intervals(15secs, 30secs, 45secs, 1min, 3mins, 5mins, 10mins, 15mins, 20mins, 30mins, 60mins, 24hrs, 48hrs and 72hrs. Then samples were transfered to brain heart infusion broth for culture and incubated for 7 days. A broth dilution test was performed and the timing of irrigants to kill the microbial cells were evaluated. Results showed that most effective concentrations of tested irrigants were ranked from strongest to weakest as follows: 100% octenisept, 50% octenisept, 5.25% Naocl and 2.5% Naocl and concluded that antimicrobial action of irrigation depends on its type and cocentration.[31]

Retomozo et al aimed to determine the concentrations and contact time required to eliminate the enterococcus faecalis in dentin cylinders of bovine teeth. Four hundred and fifty dentin cylinders(5mm in diameter ,4 mm in height with 2-3 mm in width of lumen) were prepared from bovine teeth. The cylinders were then divided into 3 groups, and 1.3%, 2.5%, or 5.25% concentration of NaOCl was applied in 5-, 10-, 15-, 20-, 25-, 30-, 35-, and 40-minute intervals for a total of 30 subgroups including positive and negative

controls. After inoculation and incubation of cylinders with enterococcus faecalis, specimens were exposed to respective irrigants and time period. Colony forming units were counted. Results obtained were :5.25% sodium hypochlorite at 40 mins showed better efficacy than other concenterations. Irrigation with 1.3% and 2.5% NaOCl for the same time interval was ineffective in removing E. faecalis from infected dentin cylinders. High concentration and long exposure to NaOCl are needed for elimination of E. faecalis contaminated dentin.[32]

Bhuva et al studied the anti enterococcus faecalis activity of 1% sodium hypochlorite with passive ultrasonic irrigation and conventional syringe irrigation in a extracted single rooted teeth. Forty eight single rooted teeth were selected and root canals were prepared with F3 protaper rotary file to the working length. The specimens were divided into four groups: group1:- conventional syringe irrigation + 1% Naocl, Group 2:- ultrasonic irrigation with 15 size + 1% Naocl, Group 3:- conventional syringe irrigation with saline, Group 4:- Not exposed to any irrigant. Enterococcus faecalis biofilm were grown on root canals and exposed to experimental irrigants. Root specimens viewed under scanning electron microscope at three levels at coronal, middle and apical third of specimens. Results showed that there were no significant differences between the conventional syringe irrigation and passive ultrasonic irrigation with 1% Naocl.[33]

Shen et al evaluated the antimicrobial effect of two chlorhexidine preparations with and without mechanical agitation (ultrasonic and sonics) in hydroxyappatite discs. The hydroxyapatite discs were coated with type I collagen and inoculated in multispecies

biofilm and incubated for 21 days. 2% chlorhexidene and CHX plus (2% chlorhexidene and wetting agents) were used in this study. The hydroxyapatite discs were immersed in testing solution and agitated for 1 and 3 mins time exposure and live /dead microorganisms estimated using confocal laser scanning microscope. Results showed that sonic activation showed highest antimicrobial effects at both the time exposures.Theyconcluded that low intensity of sonic activation does not improve the antimicrobial effect of chlorhexidene preparations.[34]

Ozdemir et al aimed to evaluate the effects of EDTA and sodium hypochlorite on enterococcus biofilm in root canal dentin of young and old individuals. Sixty single rooted human teeth (n=30 from young patients <30yrs from old patients > 60 yrs) were selected. Specimens were decoronated and apical third of root were discarded,only middle part of root used in the study . Gates glidden bur s were used to instrument the root canal of specimens. From each age groups,specimen were subdivided into four subgroups .They were: Group 1 :Treated with 10ml of 17%EDTA for 10 mins and 10ml of 2.5% sodium hypochlorite for 10 mins, Group2: Treated with EDTA alone. Group 3: Treated with Naocl alone, Group 4: Treated with 10ml sterile phosphate buffered saline for 10 mins ,as control.After irrigation half of the specimens were cultured and colony forming were counted.The remaining used to quantitative dead/ live cells in confocal laser scanning microscope.Results showed that both EDTA and Naoclirrigantsshowed equal efficacy in removing enterococcus faecalis from root canal dentin but higher number of bacteria was found in old age group than young group.Itwas concluded that elderly population were very susceptible to enterococcus faecalis biofilm in root canal dentin.[35]

Rocas and Siqueira et al conducted a invivo study comparing the antimicrobial effectiveness of 2.5% sodium hypochlorite and 0.12% chlorhexidenein necrotised pulps with apical periodontitis. The sampling were taken at following ways, such as S1: before chemomechanical preparation of root canal, S2: after chemomechanical preparation with 2.5% sodium hypochlorite. All samples were evaluated using polymerase chain reaction and bacterial strains were identified using closed ended reverse capture checker Board approach. Results showed that S1 samples were positive for bacterial species but not for fungi .Both the irrigants(sodium hypochlorite and chlorhexidene) showed efficient removal of all bacterial species.[36]

Ordinola – Zapata et alconducted a study about the biofilm removal capability of 6% sodium hypochlorite utilizing four different irrigation techniques. In this study fifty dentin specimens(2*2)were prepared from bovine teeth and attached in the Hawley 's orthodontic appliances of one volunteer to biofilm grown on it. Then the dentin specimens were incorporated in the apical third of root of single rooted human teeth which were already and sectioned for the study. Specimens were divided into four groups Group 1: Conventional needle irrigation using double side-vented needles. In this technique, the needle was inserted until 2 mm from the apex. Then, 1 mL of NaOCl was applied using a flow rate of 1 mL per 10 s and was left in root canal for 20 s, this procedure was repeated two more times for a total period of 1 min of treatment. Group 2: Endoactivator: 1 mL of NaOCl was applied at the apical third followed by the sonic activation of the irrigant using a yellow Endoactivator (15.02) tip for 20 s. This procedure (irrigation/sonic activation) was repeated two more times for a total period of 1 min of sonic treatment. The Endoactivator tip was inserted until 2 mmfrom the apex. Group 3: Passive ultrasonic irrigation (PUI): In this technique, a similar procedure was applied in the same manner

described for the Endoactivator group, but an Irrisafe file 20.00 (SatelecActeongroup, Merignac, France) was used in conjunction with a Satelec P5 suprasson ultrasonic unit (Suprasson P5; SatelecActeongroup) at a power setting of 4. Group 4: Laser-activated irrigation (LAI). An Er: YAG laser with a wavelength of 2940 nm (Fidelis; Fotona) was used to irradiate the root canals using a 12-mm 400-lm quartz tip. The laser operating parameters were: 20 mJ per pulse, 0.30 W, 15 Hz and 50 ls pulse duration. An endodontic fibre tip (PIPS; Fotona) was placed into the coronal access opening of the access cavity. One millilitre of NaOCl was applied and activated for 20 s. This procedure was repeated two more times. Group 5: Control, the initial irrigation procedures were similar to group 1, except that distilled water was used for the initial and final irrigation procedures. In this technique, 4 mL of distilled water was initially used for 4 min. For the final irrigation purposes, 1 mL of distilled water was applied using a flow rate of 1 mL per 10 s and was left in the root canal for 20 s. This procedure was repeated two more times for a total period of 1 min of treatment. Group 5: Control, the initial irrigation procedures were similar to group 1, except that distilled water was used for the initial and final irrigation procedures. In this technique, 4 mL of distilled water was initially used for 4 min. For the final irrigation purposes, 1 mL of distilled water was applied using a flow rate of 1 mL per 10 s and was left in the root canal for 20 s. This procedure was repeated two more times for a total period of 1 min of treatment. They concluded that laser activated 6% sodium hypochlorite showed better antimicrobial activity followed by passive ultrasonic irrigation.[37]

Morgental et al compared the antibacterial effect of 6% and 1% sodium hypochlorite,Qmix, 2%chlorhexidene and 17%EDTA in the presence or absence of dentin powder. The dentin powder were collected from five bovine single rooted teeth and mixed with the enterococcus faecalis suspension in eppendorf tubes. Then the experimental

solutions were added to eppendorf tubes. Suspensions were collected from time intervals at 10,30 secs & 1 mins and inoculated in brain heart infusion broth . Colony forming units were counted. Results obtained were: after 10 seconds of contact, 6% NaOCl showed the lowest counts and was significantly different from the negative control .After 30seconds, 6%NaOCl obtained null values. Dentin inhibitory effect on 6% NaOCl at (10seconds),1%NaOCl(10seconds 1minute), and QMiX(10 seconds and 1min).[38]

P. Neelakantan et alconducted an Enterococcus faecalis study investigating the effect of three irrigation protocols activated by three different methods. 280 single rooted teeth were selected and instrumented using rotary nickel titanium rotary files. Specimens were divided into three groups(n=80) and one control group (n=40), according to the irrigation protocol. Groups were :Group 1:- 1:1 mixture of 6% Naocl and 18% etidronic acid, Group 2:- 3% Naocl and 17% EDTA, Group 3:- 3% Naocl + 1% EDTA + 3% Naocl. Groups weresubdivided into subgroups suh as (n=20) A) no activation B)ultrasonic activation C) diode laser D) Er: YAG laser. After application of irrigation methods, specimen were subjected to viability staining for viewing under confocal laser scanning microscope to evaluate live / dead proportion of enterococcus faecalis and radicular sampling were taken for analysing colony forming units. Results obtained in this study was: Diode laser and Er YAG laser were more effective in removing E.faecalis biofilm than the ultrasonic activation and conventional syringe irrigation. No significant difference found between Naocl + etidronic acid and Naocl – EDTA – Naocl.[39]

Oliviera et al conducted a study on different concentration of sodium hypochlorite(1%,2.5%& 6%) and chlorhexidine(0.12%,0.2%&2%) against enterococcus

faecalisbiofilm. Microfiber plate wells containing enterococcus faecalis biofilm exposed to different concentrations of experimental solutions at different time intervals (1, 3,& 10 mins). The resultant biofilm was observed for optical density under microplateabsorbance readerfor evaluation. Results obtained in this study was that the three CHX solutions were not significantly different from the negative control group in reducing the biofilm biomass.In contrast, after 10 mins of exposure to the NaOCl, the biofilm was not significantly different from the positive control without biofilm.At all time exposures,NaOCl solutions were significantly more effective than any of the CHX solutions tested. They concluded that none ofthe concentrations of chlorhexidene showed efficient removal of enterococcus faecalisbiofilm. 1% sodium hypochlorite showed efficient removal biofilm mass in titre wells.[40]

Wong et alinvestigated the effect of two concentrations of sodium hypochlorite on infected root canal dentin and also estimated the extent of disinfection in to the dentinal tubules.In this study, nine single rooted extracted teeth were selected and two specimens irrigated with sterile saline were used as negative control.0.5% and 3% of Naocl were used for this study. Decoronation of specimens were done and gates glidden burs used in coronal third preparation and the remaining part of root canal were instrumented with profile rotary instruments of apical size 30. Then the roots were split into two halves buccolingually . Dual species such as Enterococcus faecalis and porphyromonasgingivalis were inoculated in root canal and incubated the specimens for 7 days. Then the specimens were treated with experimental irrigants and live /dead staining preparations done in all specimens prior to confocal laser scanning microscope imaging. Results obtained were : Both the concentrations showed equal efficacy in first 0.1mm of depth of root canal dentin. In the depth range of 0.1mm-0.3mm in to dentinal tubules, 3% concentration

showed better results than 0.5% concentration naocl. In all the layers of dentinal tubules,the antibacterial effect of sodium hypochlorite is dependent on the available concentration of solution.[41]

De Almeida et al compared the two endodontic irrigants such as 2.5% calcium hypochlorite and 2.5% sodium hypochlorite with and without ultrasonic activation against enterococcus faecalis biofilm in infected bovine root dentin.Sixty bovine single rooted teeth were selected, decoronated and instrumented with k files up to apical size of 45. Then root specimens were autoclaved , inoculated with enterococcus faecalis and incubated for 30 days. Specimens were divided in to ten groups such G1:No treatment,G2: distilled water,G3:2.5% Naocl, G4: 2.5% Ca(ocl)2,G5:2.5% Naocl with ultrasonics,G6: 2.5% Ca(ocl)2 with ultrasonics.After irrigation with respective irrigants, colony forming units were formed ,evaluated and counted the units. Results obtained In this study were calcium chloride with ultrasonic showed lowest contamination compared to other experimental irrigants with no significant differences statistically among group3,4,5.[42]

Rita et al ,compared anti E.faecalis efficiency of sodium hypochlorite, chlorhexidene and ozone either alone or in combination of root canal irrigants. In this study concentration of irrigant s used were sodium hypochlorite at 1%,3%and 5%. Chlorhexidene at 0.2%,2%.and Ozone gas. The strains of experimental organisms were made to inoculated with the specimens and observed under flow cytometry to determine the mechanism of action of irrigants on microorganisms. The results were observed that combination of

chlorhexidene 2% and ozone for 24 secs showed advantageous when treating infected root canals.[43]

Tianfenget al investigated the antibacterial efficiency of various root canal irrigants on young(1day) and old(3weeks). Here 24 teeth were selected and roots of the specimens were cut in to cylindrical shaped specimens of 6mm in length. The root canals of prepared specimens were instrumented using gates glidden bur and split in to two halves buccolingually .All specimens were infected with enterococcus faecalis and the biofilm formation were allowed to form on it in respect to different time intervals such as one day and 3weeks duration. The endodontic irrigants such as 2% chlorhexidene, 2% sodium hypochlorite, and 6% sodium hypochlorite were used. The specimens were divided in to 3 groups (n=12). 12 specimens in each group were subdivided equally for two types of biofilm. Then experimental irrigants were applied on different time intervals such as 1,3 and 10 mins. After irrigation, specimens were viewed under confocal laser scanning microscope for determining the live and dead proportions of enterococcus faecalis in the biofilm. The results of the study showed that the proportion of dead cells in the young biofilm were more than the dead cells in old biofilm. The killing effect of irrigants were rapid in the first 3 mins and then the killing effect were slowed down after 3 mins. The 6% sodium hypochlorite showed better results than other irrigants used. Statistically there were no significant differences between 2%sodium hypochlorite and 2% ch!orhexidine.[44]

Salian et al conducted a study about comparison of three different irrigating solution such as antibiotic containing irrigant, 2%chlorhexidene and chlorhexidene combining with cetrimide on enterococcus faecalis infected dentin. In this study, forty teeth specimens

were infected with enterococcus faecalis for 28 days. Groups in this study were group A : Coamoxiclav/citric acid /mage 80, group2: 2% chlorhexidene gluconate, group3: chlorhexidene and cetrimide. After irrigation and culturing in the culture media, specimens were incubated at 37degree Celsius and colony forming units were counted. The results obtained were group A showed better antibacterial effect than other groups. Group 3 showed better results than group 2 containing 2% chlorhexidene.[45]

DrRuqshanAnjum et al conducted a study and compared chlorhexidene, Oxum and ozonated water. In this study forty single rooted teeth with root canals were contaminated with the enterococcus faecalis and incubated at 37 degree for 7 days. After incubation of 48hrs ,the root canals were instrumented up to 80 size k files and then irrigated with above said experimental irrigating solutions. They observed that chlorhexidene reduced the number of E faecalis than oxum and ozonated water.[46]

Ruiz – linares et al evaluated the antimicrobial activity of 2.5% sodium hypochlorite, 2% chlorhexidine, 2% Alexidine and 0.2% cetrimide on mature polymicrobial root canal biofilm on human dentin using confocal laser scanning microscope. Sterile twenty eight human dentin,specimens(4×4×0.7mm) were prepared and infected with microbial samples collected from infected roots of three volunteers. The specimens were incubated for 21 days and exposed to antimicrobial agents. After staining specimens were observed under confocal laser scannng microscope. Results showed that 2.5% Naocl efficiently removes polymicrobial mature biofilm on dentin specimens than other irrigants used. Cetrimide synergises the antibacterial activity of alexidine and chlorhexidine.[47]

Sevnic et alcompared the contact time,concentration and temperature application on antibacterial activity of sodium hypochlorite against enterococcus faecalis. Antibiotic discs were used in this study and groups were divided in to six groups such as Group1:25degree C,5%,1min, Group2: 25degree C,2.5%,5mins, Group3: 37degree C,5%,1 mins, Group4: 37degree celsius,2.5%,5mins, Group5:45degree celsius,5%,1min, Group6: 45degree celsius ,2.5%,5min. After irrigation, antibiotic discs were placed in 3 ml of sodium thiosulphate solution for neutralizing the Sodium hypochlorite and inoculated in sheep blood agar plates. Colony forming units were counted. They concluded that increase in concentration, contact time and temperature of sodium hypochlorite increased antimicrobial efficiency of sodium hypochlorite.[48]

Giardino et al compared the antienterococcusfaecalis activity of 5.25% NaOCl, Hypoclean(NaOCl-based irrigant modified with surfactants) and Chlor-Xtra ((<6% NaOCl solution modified with Triton X100, proprietary surface modifiers and alkylating agents) at 20 °C and 45 °C in bovine root dentin. One-hundred-and-seventy dentin tubes prepared from bovine maxillary incisors were infected with Enterococcus and for 21 days. The specimens were divided into eight groups: 1. 5.25% NaOCl 20 °C; 2. Hypoclean 20 °C; 3. Chlor-Xtra 20 °C; 4. 5.25% % NaOCl 45 °C; 5. Hypoclean 45 °C; 6. Chlor-Xtra 45 °C; 7. positive control; 8. negative control. Dentin chips collected from dentin specimens by using round burand inoculated in brain heart infusion broth. After culturing, colony forming units were counted. Modified sodium hypochlorite solutions showed least numbers of colony forming units when compared to sodium hypochlorite alone. At 45

degree Celsius, modified Naocl solutions showed greater antimicrobial effect than other tested irrigants.[49]

Yang et al conducted a study on infected dentin model evaluating the effect of source of biofilm bacteria on their susceptibility in dentinal tubules to chlorhexidene and 2% sodium hypochlorite. Monospecies biofilm of enterococcus faecalis strains VP3-181 and Gel 31 were used. The centrifugation process were used for the introduction of strains into the dentinal tubules in infected dentin models. Sixty dentin blocks were prepared and infected with above said strains of microorganisms. They were first divided into 5 groups (3 plaque groups and 2 E. faecalis groups) and further divided into 1-and 3-weeks biofilm groups (6 dentin specimens in each). After incubation for 1 and 3 weeks and 3 mins exposure of irrigant solution, cell viability staining applied on all the specimens. Then confocal laser scanning microscopy were used to propotionate the live / dead microorganisms in dentin specimens.Results showed that No differences in the susceptibilities to the disinfecting agents of the 3 multispecies biofilms were detected; 2% NaOCl was more effective against multispecies biofilms in dentin than 2% CHX.[50]

Pladisai at al evaluated the different disinfection protocols on enterococcus faecalis infected dentin. Forty eight single rooted human mandibular premolars with large canals were selected and instrumented up to 60 size. Then specimens were infected with enterococcus faecalis and incubated for 21 days Specimens were grouped in to five groups according to disinfection protocols were followed.Groups were G1:mechanical instrumentation (circumferential filing with 70,80,90 sizes)and 2.5% Naocl.G2:Rotary instrumentation and 2.5% Naocl,G3: passive ultrasonic irrigation intermittently and 2.5%

Naocl, G4:Irrigated with normal saline, G5: No treatments were done.Dentin chips were collected using peeso reamers and deposited ineppendorf tubes. The samples were cultured on blood agar plates. Colony forming units were formed and counted.Results showed that MINshowed least bacterial growth compared to other groups.[51]

Alaa et al investigated antienterococcusfaecalis efficacy of 5.25% sodium hypochlorite on isolated enterococcus faecalis biofilm from single rooted canal. 200 single rooted teeth were instrumented with reamers of size(15-55) and then irrigated with 5.25% sodium hypochlorite. From each root canals sampling were taken before and after application of 5.25% sodium hypochlorite and cultured in agar plates. Colony morphology, Gram stain, biochemical tests and inoculated in selective differential media to identify the bacterium. Antibiotic susceptibility test done using BD Phoenix system. Sixteen isolates of enterococcus faecalis were identified before treatment.Then after application ,eleven isolates were eradicated whereas only 5 isolates were resistant to sodium hypochlorite.In the antibiotic sensitivity test,before application of sodium hypochlorite, 93.75% of enterococcus faecalis were susceptible to nitrofurantoin,amoxicillin and linezolid (62.25%), Vancomycin and teicoplanin(50%) but after application of sodium hypochlorite resistant rate for nitrofurantoin(80%) and for other antibiotics (60%). This study concluded that elimination of enterococcus faecalis by sodium hypochlorite was excellent and confirmed by the Phoenix system.[52]

MicoogullarKurt et al compared the post operative pain and periodontal healing among the single and multiple visit root canal therapy when chlorhexidene used as a final irrigant. Here, ninety asymptomatic maxillary anterior teeth with periapical lesions were selected. Root canal prepared using step back techniques by k files and 2.5% Naocl and 5% EDTA. Then 2% chlorhexidene were used as final irrigant. In multiple visit, calcium hydroxide

paste used as an intra canal medicament. All patients were examined radiographically and clinically for 24 months.In this study,chlorhexidene was used as a final irrigant and calcium hydroxide used as a final dressing. They concluded that single visit endodontic therapy with chlorhexidene as a final rinse showed an acceptable post operative periapical healing compared with multiple visit root canal treatment with calcium hydroxide as an intracranial medicament.[53]

Mohamed et al compared the efficacy of sodium hypochlorite with active and passive irrigation techniques against enterococcus faecalis biofilm in the lateral canals of simulated root canal models. Using transparent resin materials with 3D printing, root canals models were manufactured. Root canal of length 18Mm and a lateral canal of 3mm in length were standardised and instrumented up to 30 size 6% taper. Biofilm were grown on the root canal specimens and experimental irrigating solutions were applied in the biofilms. Then the models were observed under fluorescence microscopy.In this study they concluded that penetration of 2.5% sodium hypochlorite into the lateral canal was better when used along with agitation techniques. Of these techniques ultrasonic agitation showed better results than sonic ,manual and passive irrigation methods.[54]

AustinYoo et aldetermined the response of sub minimal inhibitory concentrations of sodium hypochlorite on biofilm growth in respect to extracellular material in biofilm. Enterococcus faecalis strains were selected according to biofilm producing ability using Crystal violet biofilm assay, such as 10 strains of strong producing,, 4 strains of intermediates,and 5 strains of nonbiofilm producers. All biofilms were made to contact with Naocl and assessed the quality of extracellular material of biofilm. Then the biofilm were evaluated using scanning electron microscopeThey suggested that due to clonal diversity among strains of enterococcus faecalis, strong biofilm producers yield more ECM production than slow producers in response to sub MIC of sodium hypochlorite.[55]

Materials and methods

The following armamentarium were used

1) Eighty extracted human single rooted tooth.

2)Diamond disc.

3)Autoclave.

4) Petri dish plates.

5) conical flasks.

6) peptone agar medium.

7) Inoculation loops.

8) Eppendorf tubes.

9) Micropipette.

10) Scanning electron microscope.

11) Laminar flow chamber.

12) 5.25% sodium hypochlorite.

13) 3% sodium hypochlorite.

14) 1% sodium hypochlorite.

15) 2% Chlorhexidine.

16) 1% trypsin.

17) Normal saline.

18) Enterococcus faecalis.

19)Ultrasonic unit(wood pecker).

20) Viable staining preparation kit.

21) 17% EDTA.

22) Confocal laser scanning microscope.

Materials and methods:

Eighty single rooted teeth with periodontal problems were extracted and stored in saline. The fractured teeth and teeth with caries were excluded. Specimens were decoronated at the cementoenamel junction and apical third of root was discarded. Only the middle portion of the root was used for the experimental study. From each specimen the cementum layer on the border of the specimens was removed using carborundum disc. From the middle third of the root, dentin discs of 6mm diameter was obtained by using a high speed rotating diamond disc along with water coolant.The specimens were kept in an ultrasonic bath for 3 mins to remove the smear layer present on the surface of the dentin specimens. The dentin specimens were autoclaved at 121^{o}C for 20 mins for sterlization. The two specimens were inoculated in brain heart infusion broth to check the sterility at 37^{o}C for 24 hrs.

Enterococcus faecalis strains were obtained and cultivated in peptone agar medium at 37^{o} C for 48 hrs. Then the medium was checked for the number of bacterial cells present in it using Mac farland method obtaining (6×10^{8}cfu/ml). In each tube 2 ml of culture were added in 3 ml eppendorf tubes(n=75). The sterile dentin samples were immersed in eppendorf tubes containing medium. The whole unit was placed in an incubator at 37^{o} C for 21 days. Fresh peptone agar medium was replaced every 72 hours.

The specimens (n=5) were used to check the growth of enterococcus faecalis biofilm under scanning electron microscope.

After incubation period dentin specimens(n=75) were grouped in to five groups each containing fifteen specimens according to the experimental irrigant groups. The groups were Group1:5.25% Sodiumhypochlorite.

Group2:3% Sodium hypochlorite+ 1%Trypsin,

Group3:1% Sodium hypochlorite+ 1%Trypsin

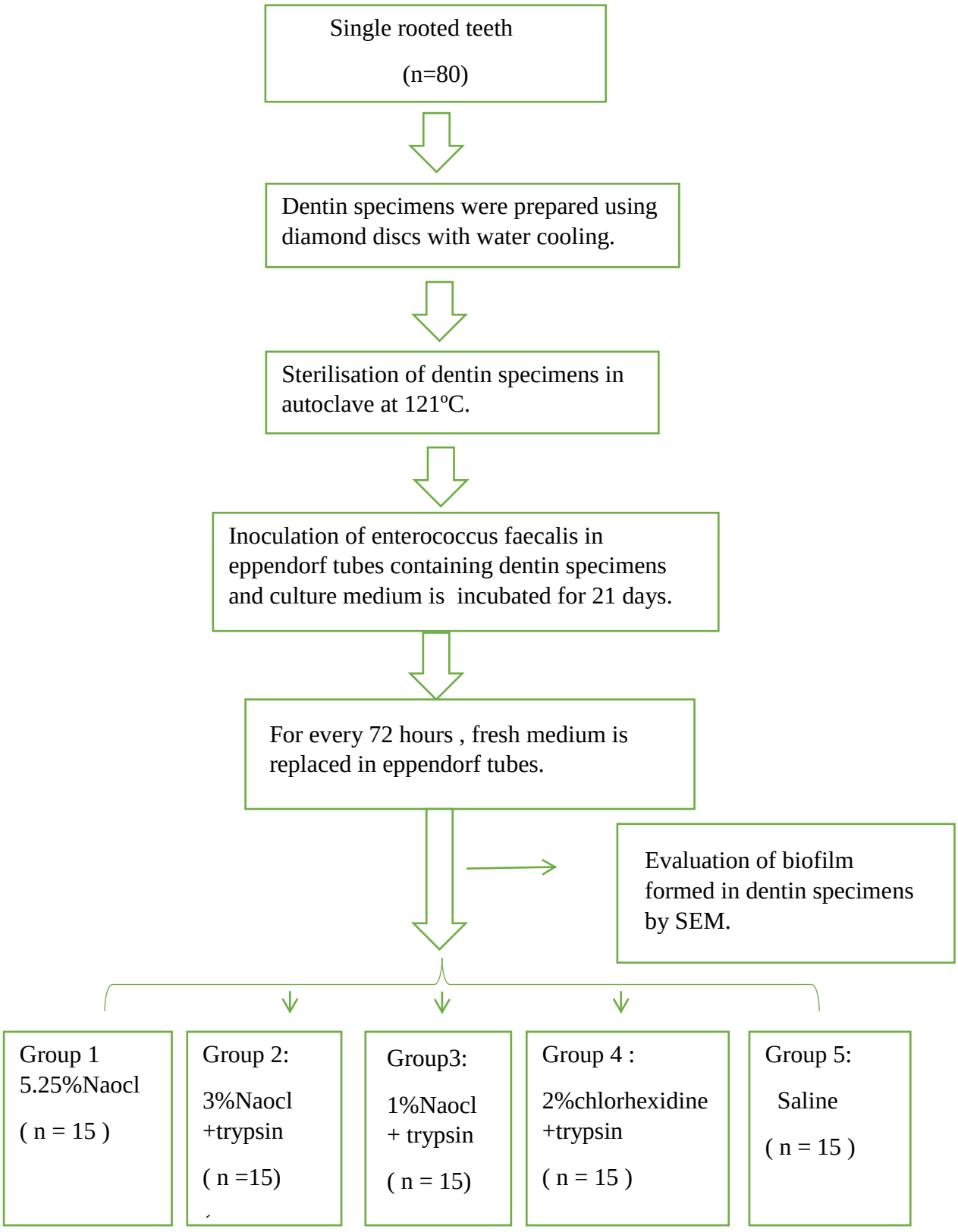
Group4:2% Chlorhexidine+1%Trypsin,

Group5:Normal saline.

Each dentin specimens were transferred to fresh eppendorf tubes containing respective irrigants and agitated by ultrasonic agitation for 2mins.

After irrigation, the staining was prepared by applying SYTO9/propiodiumiodide. Then the samples were viewed under confocal laser scanning microscopy for checking viability of enterococcus faecalis and to quantify live / dead organisms in the biofilm on dentin specimens. This viability staining kit has the property to differentiate live or dead organisms.The live organisms take the stain of green colour(SYTO9) and the dead organisms take the stain of red colour(PI).

Images were analysed under Bioimage j software for estimating the mean percentage of dead cells proportion(Red flouresence). The statistical analysis was done .


Single rooted teeth
(n=80)
Dentin specimens were prepared using diamond discs with water cooling.
Sterilisation of dentin specimens in autoclave at 121ºC.
Inoculation of enterococcus faecalis in eppendorf tubes containing dentin specimens and culture medium is incubated for 21 days.
For every 72 hours , fresh medium is replaced in eppendorf tubes.
Evaluation of biofilm formed in dentin specimens by SEM.
Group 1
5.25%Naocl
(n = 15)
Group 2:
3%Naocl
+trypsin
(n =15)
Group3:
1%Naocl
+ trypsin
(n = 15)
Group 4 :
2%chlorhexidine
+trypsin
(n = 15)
Group 5:
Saline
(n = 15)

Viability staining done in all dentin specimens.

Viewed under Confocal Laser Scanning Microscope.

Fig 1 : Dentin specimens

Fig 2 :Eppendorf tubes with dentin specimens and culture medium

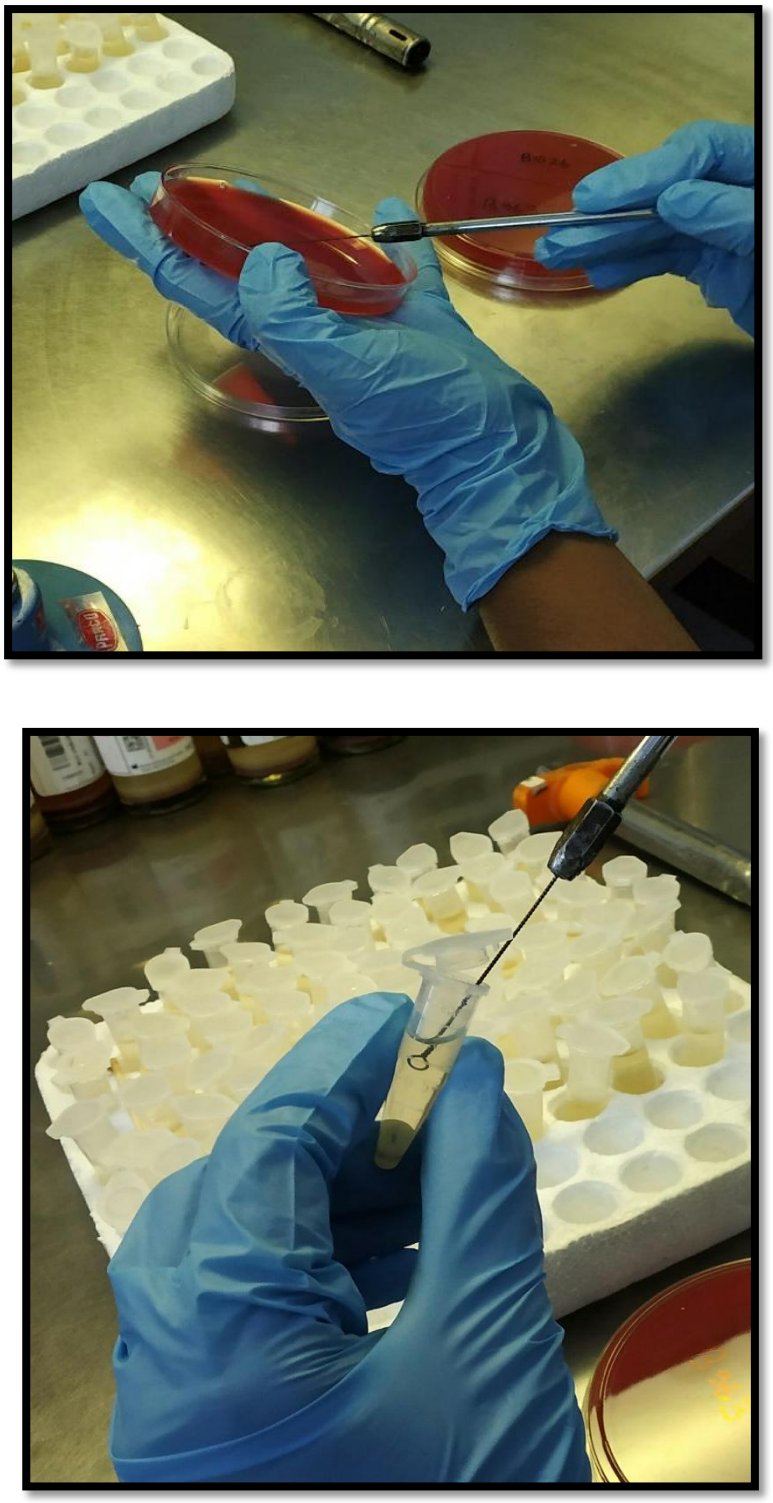


Fig 3: Inoculation of enterococcus faecalis in dentin specimens using sterile inoculatin loop.

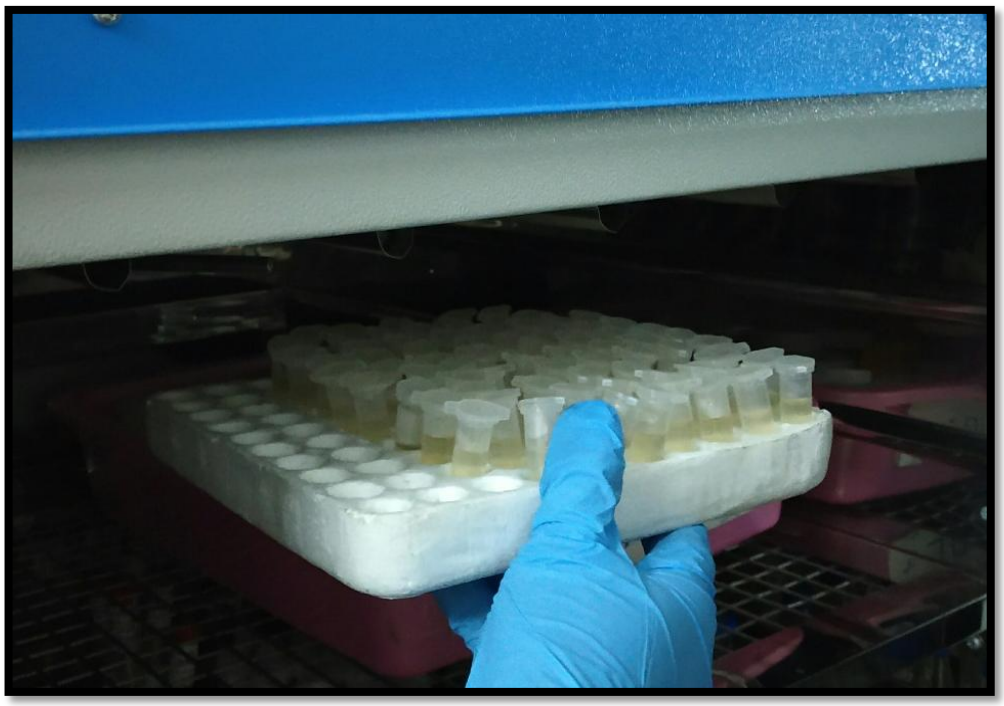


Fig 4: Dentin specimens were incubated at 37^0C for 21 days.

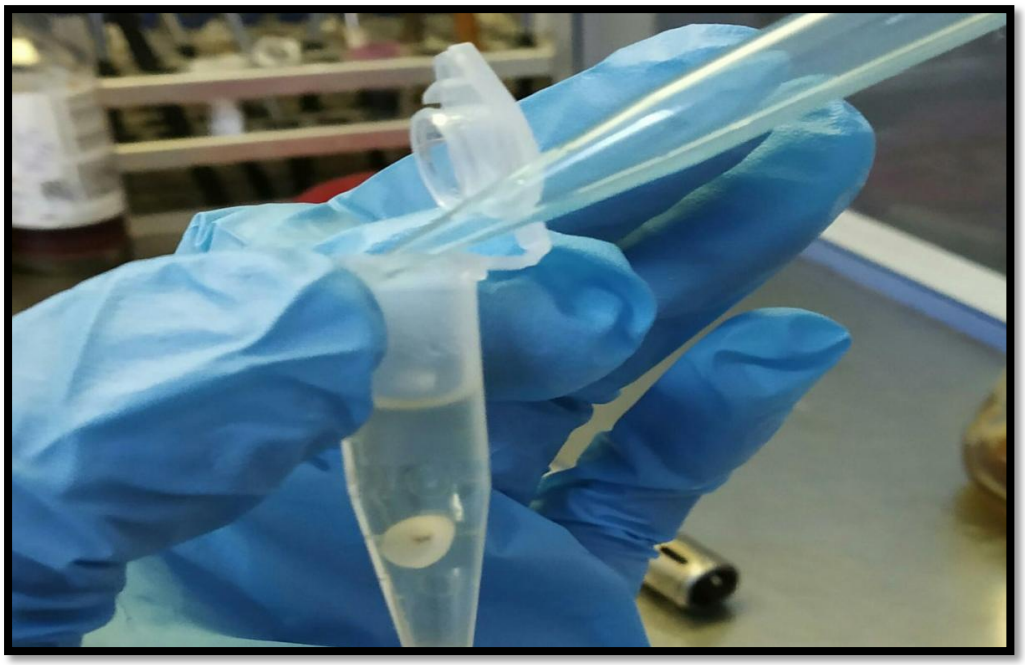
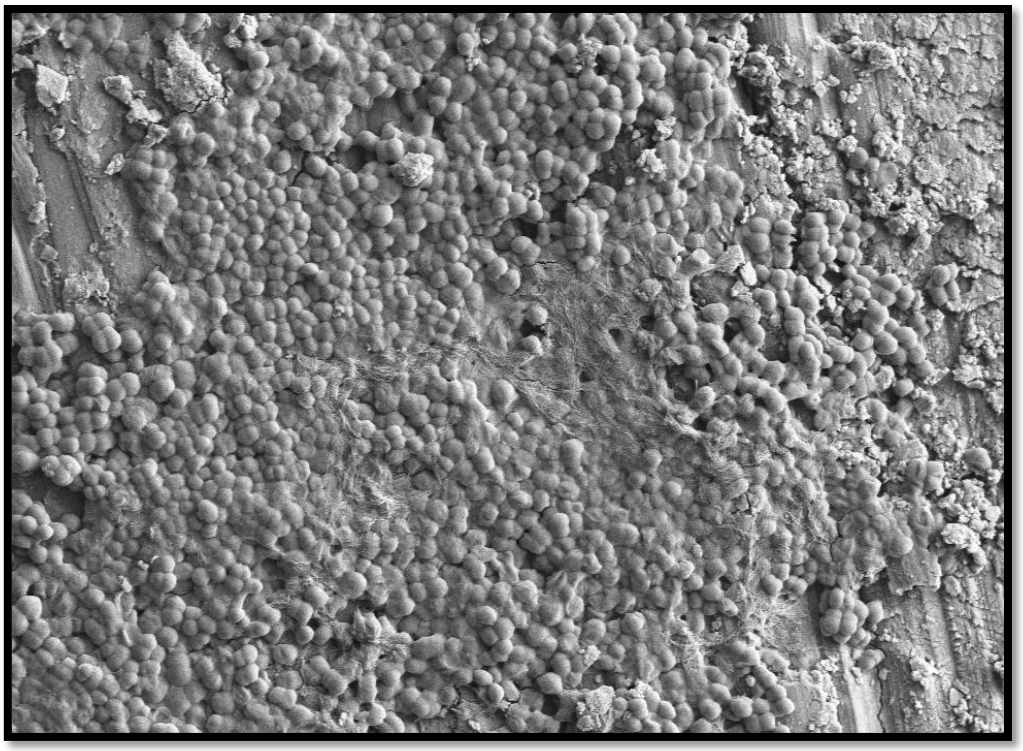


Fig 5: For every72 hrs fresh culture medium was replaced

Fig 6: Scanning electron microscope



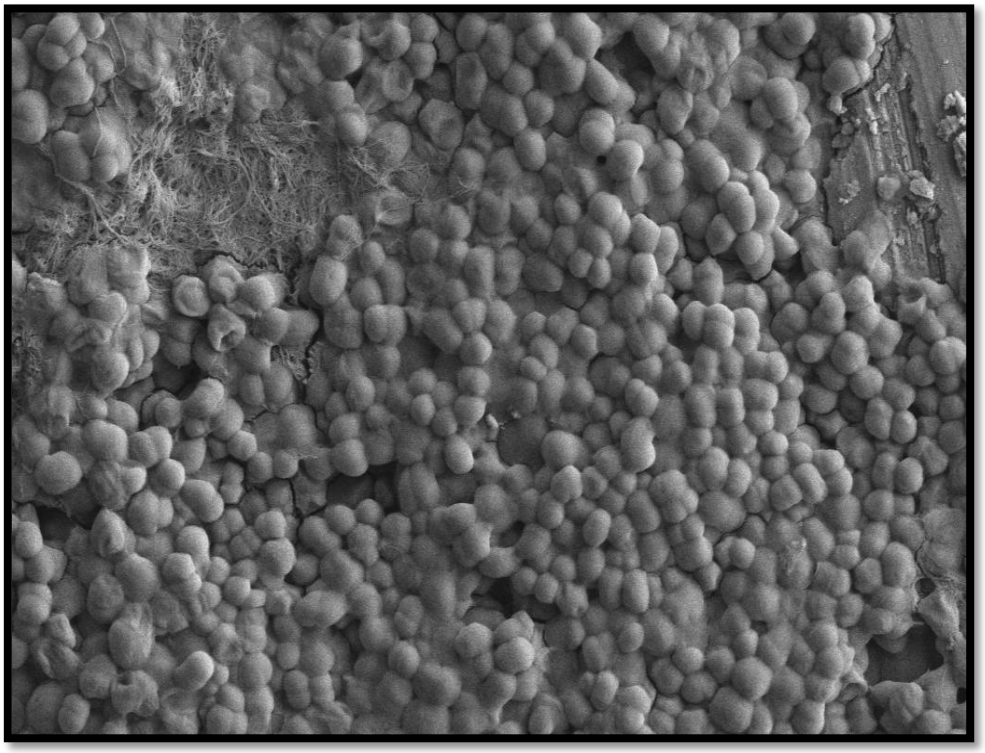


Fig 7: verification of biofilm formation in scanning electron microscope.

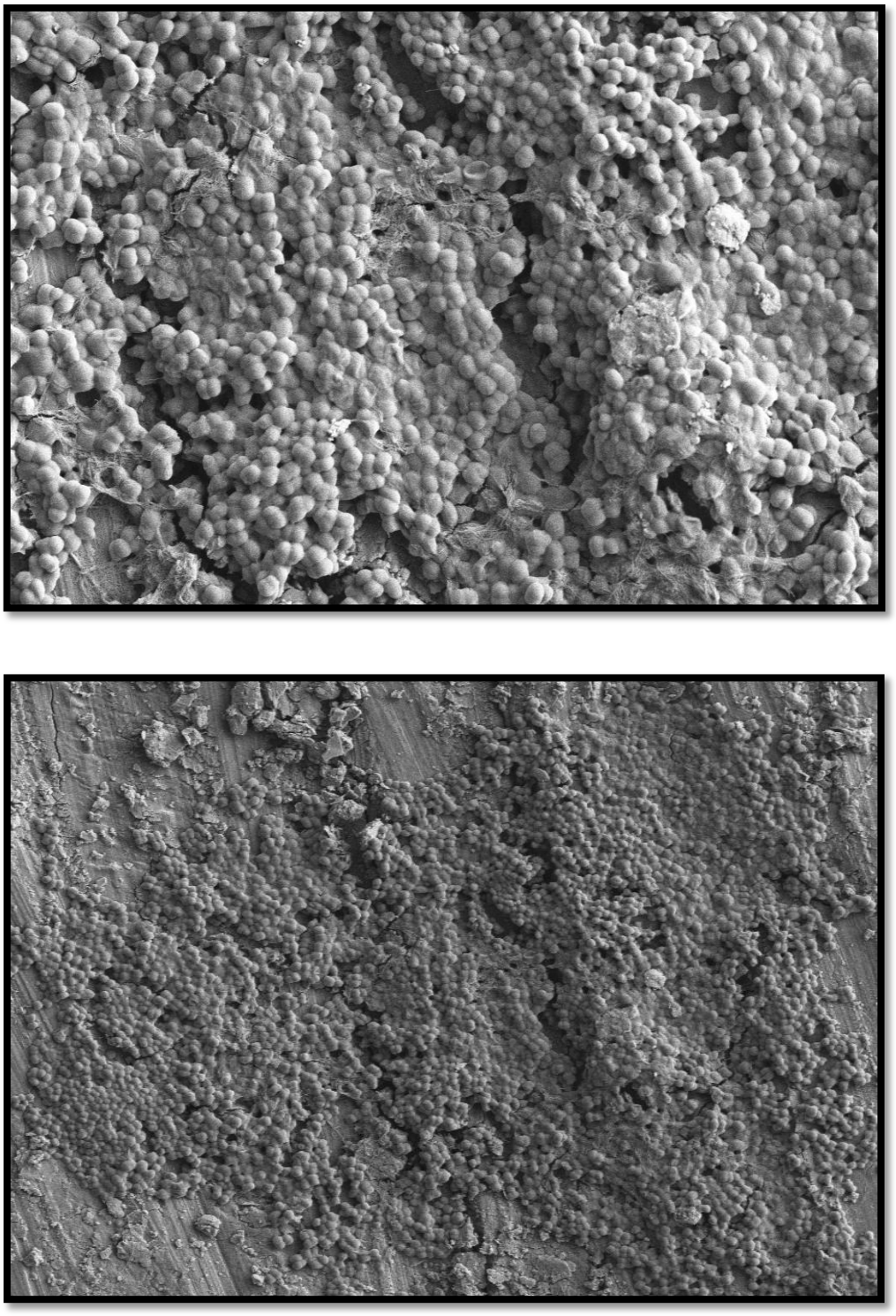


Fig 8 : Verification of biofilm formation in scanning electron microscope

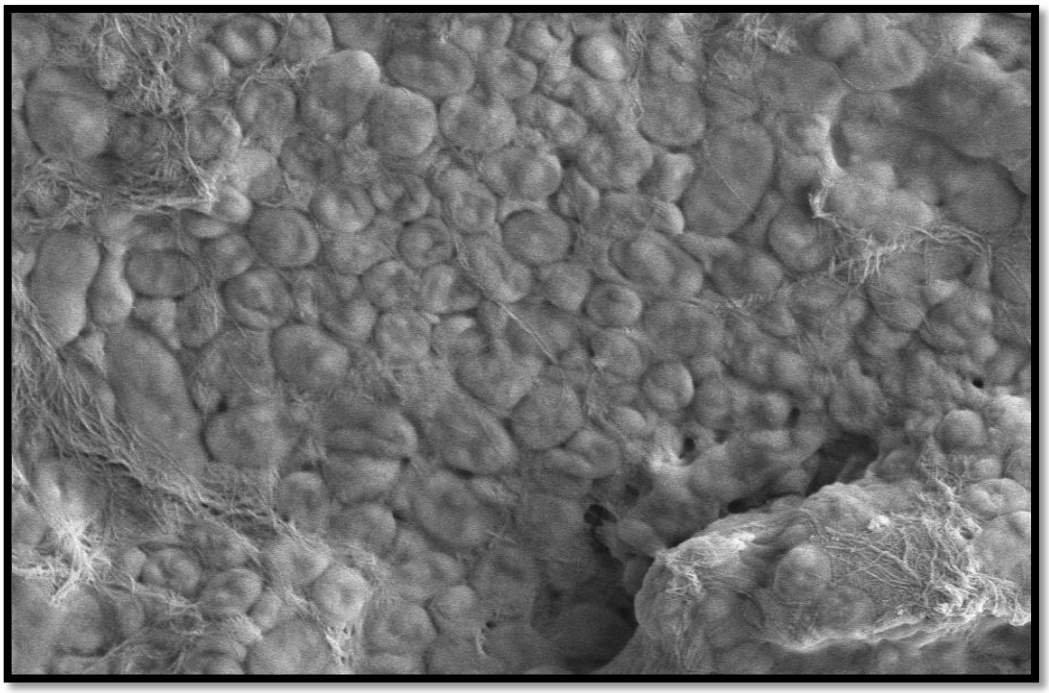


Fig 9: Verification of biofilm formation in scanning electron microscope

Fig 10: Experimental irrigants

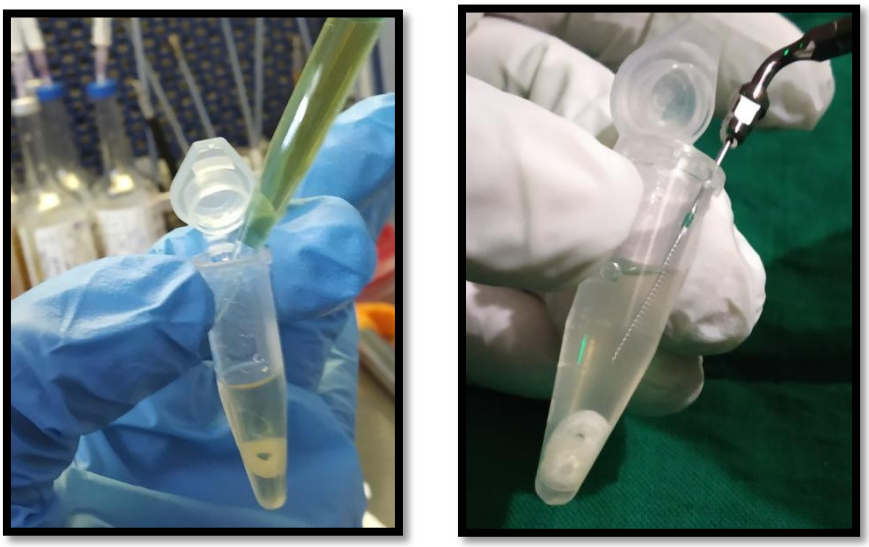


Fig 11: Application of irrigants and ultrasonic agitation on dentin specimens

Fig 12: Confocal laser scanning microscope

RESULTS

The results of the present study showed that all groups exhibited equal antibacterial effect except group5:(Saline- negative control). There was no significant difference among groups 1,2,3,4.(Pvalue>0.05). Group 1,2,3,4 showed significant difference when compared to Group 5 (P<0.05).

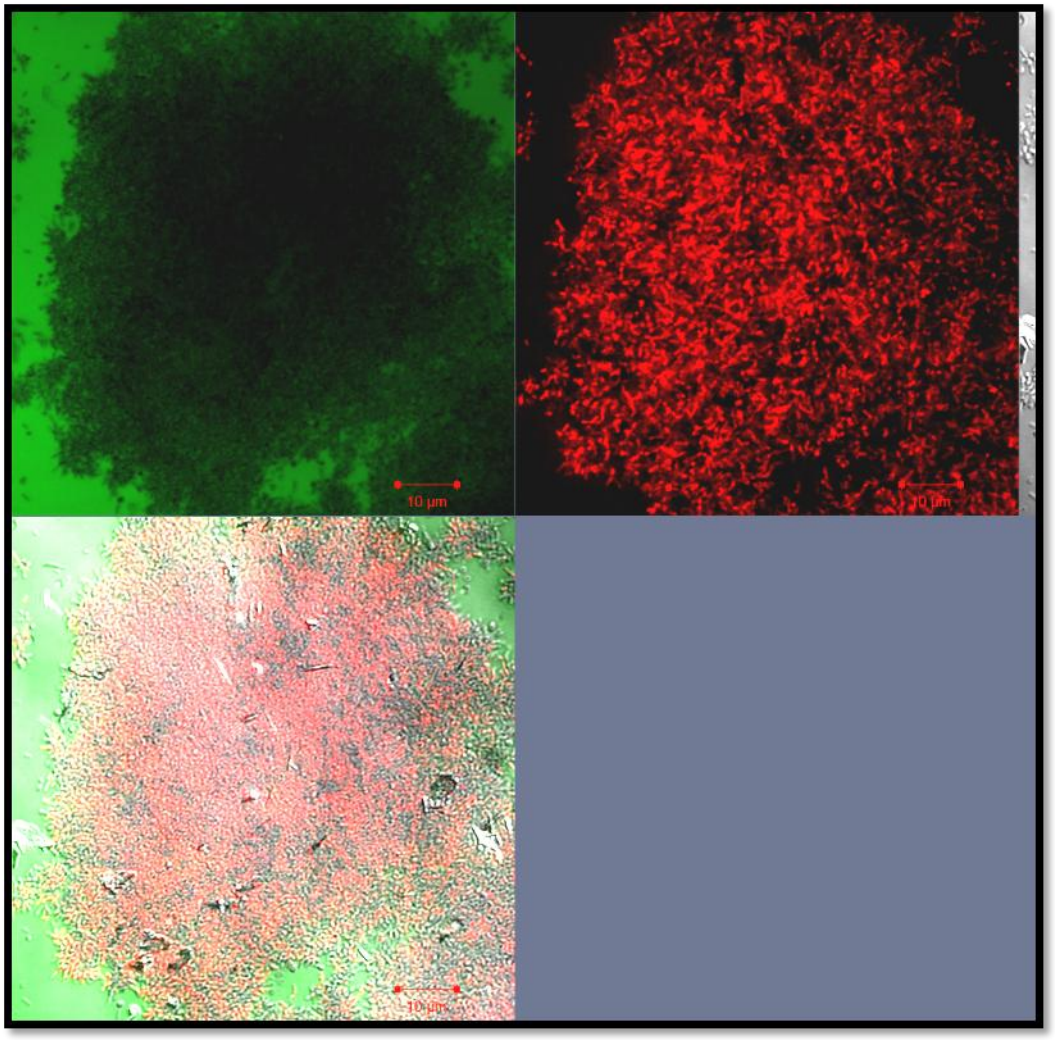


Fig 13:Confocal laser scannin image(CLSM) of dead and live enterococcus faecalis in Group1

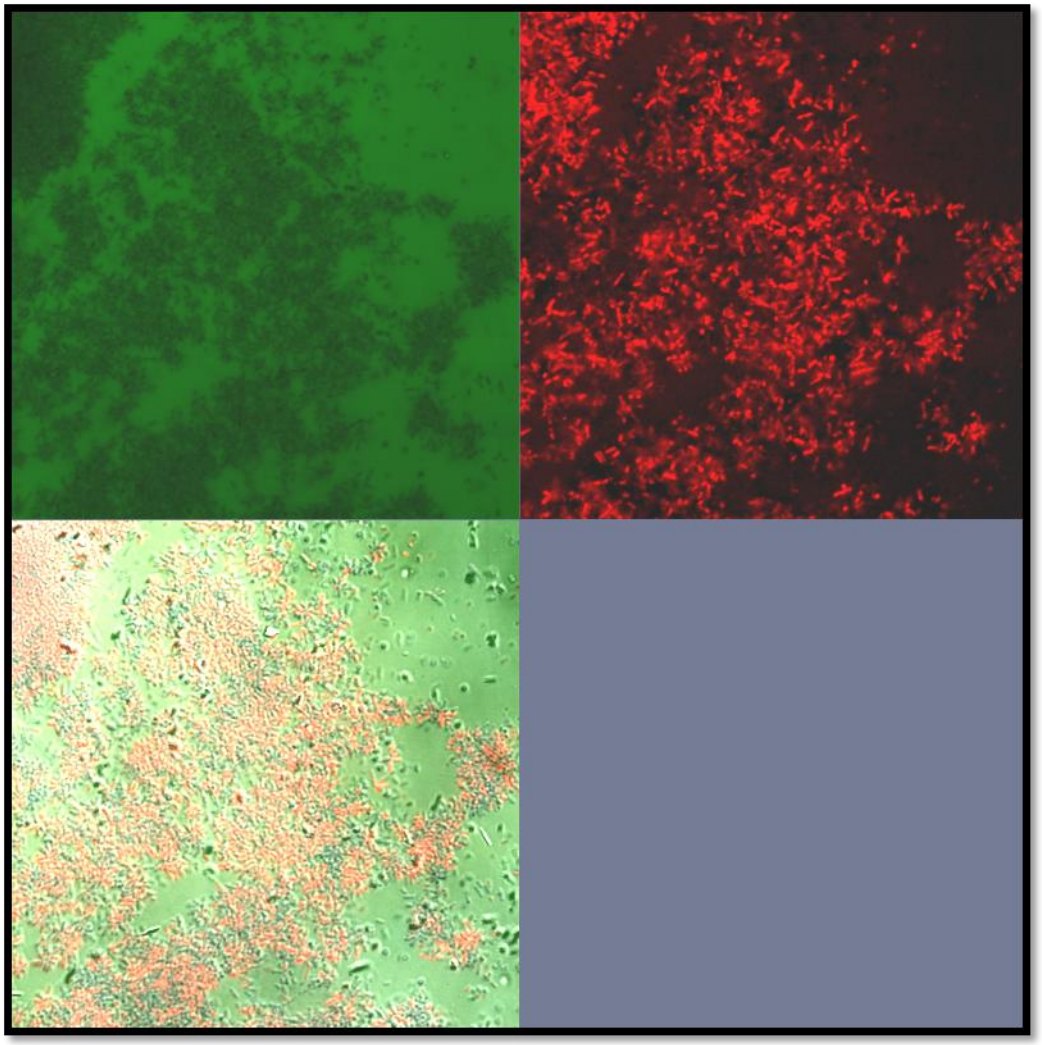


Fig 14: CLSM image of dead and live enterococcus faecalis in Group2

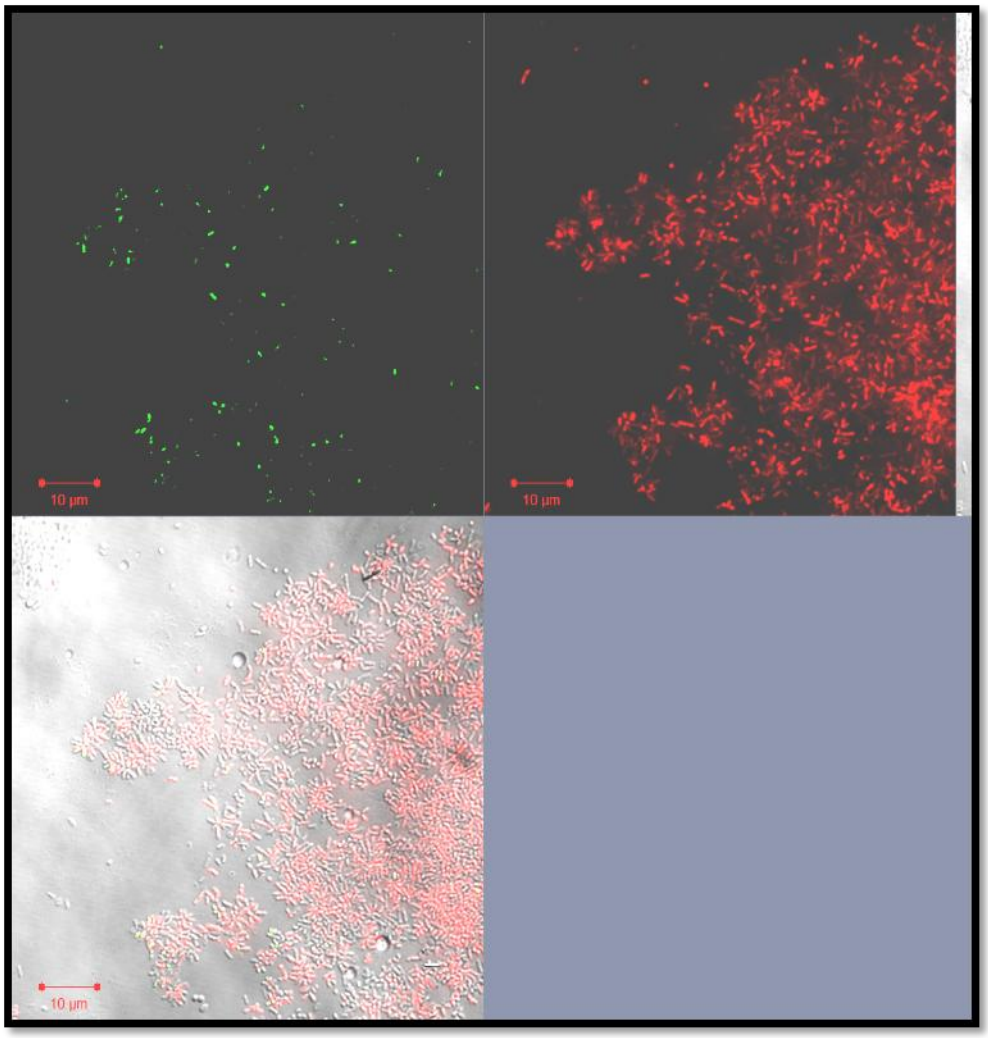


Fig 15: CLSM image of dead and live enterococcus faecalis in Group3.

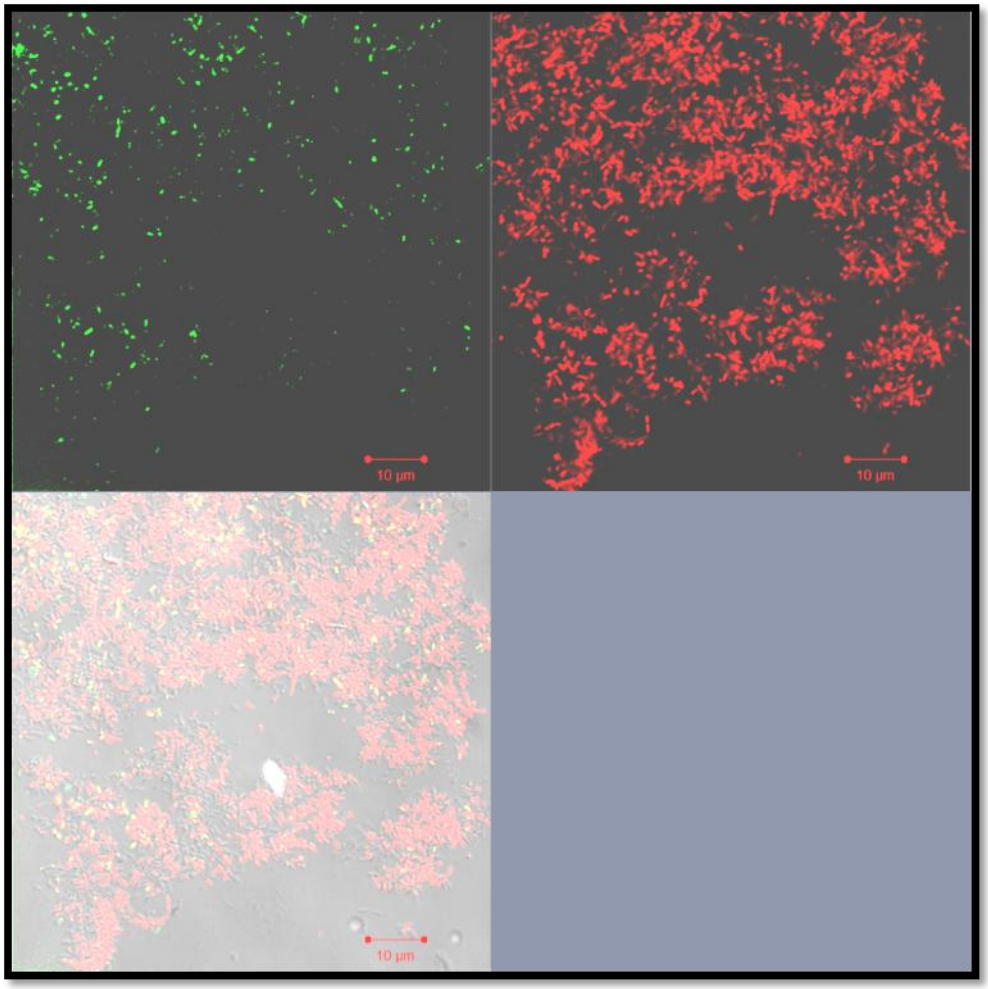


Fig 16: CLSM image of dead and live enterococcus faecalis in Group4

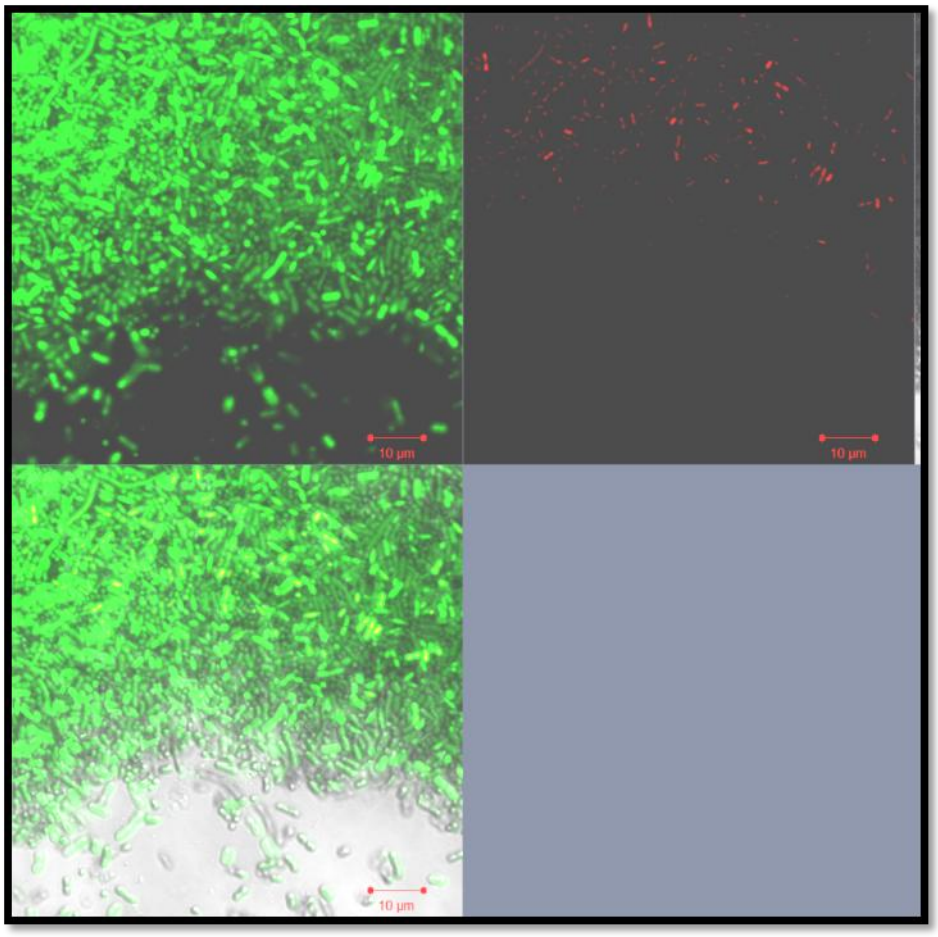


Fig 17: CLSM image of dead and live enterococcus faecalis in Group5

Descriptives

Values

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Group1 (5.25% NAOCL+1 % Trypsin)	15	90.0667	4.11386	1.06219	87.7885	92.3448
Group2 (3% NAOCL+1 % Trypsin)	15	89.9333	5.31126	1.37136	86.9921	92.8746
Group3 (1% NAOCL+1 % Trypsin)	15	86.8667	5.78010	1.49241	83.6658	90.0676
Group4 (2% CHX+1% Trypsin)	15	88.8000	3.96773	1.02446	86.6027	90.9973
Group5 (Normal Saline)	15	11.8667	3.68136	.95052	9.8280	13.9053
Total	75	73.5067	31.37583	3.62297	66.2877	80.7256

Table1: Mean, standard deviation of dead cell percentage.

ANOVA

Values

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	71339.013	4	17834.753	826.923	.000
Within Groups	1509.733	70	21.568		
Total	72848.747	74			

Table2: Anova for mean dead cell percentage

Multiple Comparisons

Dependent Variable: Values

Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group 1	2.00	.13333	1.69578	1.000	-4.6151	4.8818
	3.00	3.20000	1.69578	.334	-1.5485	7.9485
	4.00	1.26667	1.69578	.945	-3.4818	6.0151
	5.00	78.20000*	1.69578	.000	73.4515	82.9485
Group 2	1.00	-.13333	1.69578	1.000	-4.8818	4.6151
	3.00	3.06667	1.69578	.377	-1.6818	7.8151
	4.00	1.13333	1.69578	.963	-3.6151	5.8818
	5.00	78.06667*	1.69578	.000	73.3182	82.8151
Group 3	1.00	-3.20000	1.69578	.334	-7.9485	1.5485
	2.00	-3.06667	1.69578	.377	-7.8151	1.6818
	4.00	-1.93333	1.69578	.785	-6.6818	2.8151
	5.00	75.00000*	1.69578	.000	70.2515	79.7485
Group 4	1.00	-1.26667	1.69578	.945	-6.0151	3.4818
	2.00	-1.13333	1.69578	.963	-5.8818	3.6151
	3.00	1.93333	1.69578	.785	-2.8151	6.6818
	5.00	76.93333*	1.69578	.000	72.1849	81.6818
Group 5	1.00	-78.20000*	1.69578	.000	-82.9485	-73.4515
	2.00	-78.06667*	1.69578	.000	-82.8151	-73.3182
	3.00	-75.00000*	1.69578	.000	-79.7485	-70.2515
	4.00	-76.9333*	1.69578	.000	-81.6818	-72.1849

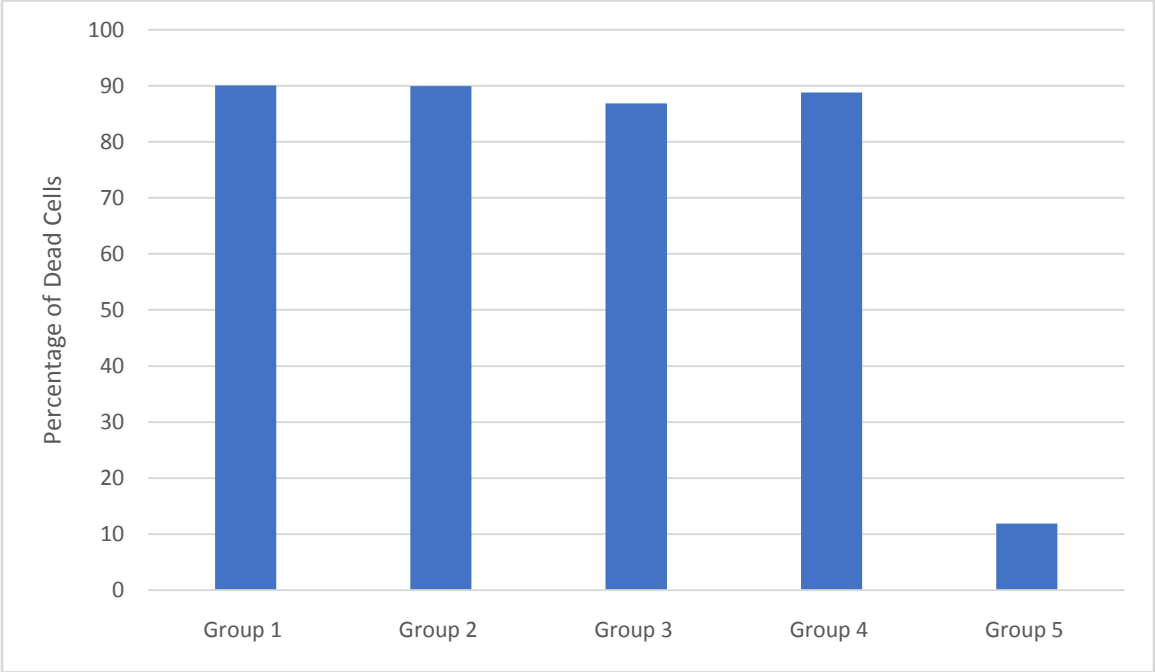
Table 3: Multiple comparison of mean dead cells within groups

Tukey HSD

Group	N	Subset for alpha = 0.05	
		1	2
5.00	15	11.8667	
3.00	15		86.8667
4.00	15		88.8000
2.00	15		89.9333
1.00	15		90.0667
Sig.		1.000	.334

Table 4 : Tukey post hoc test for mean dead cells

BAR DIAGRAM



Bar Diagram shows the mean percentage of dead cells among the groups

Discussion:-

The success of endodontic treatment is largely dependent on complete debridement of the root canal system and eradication of microbes.[1] The microbes present in a biofilm enhances the resistance to antimicrobial agents than the cells present in a planktonic state.[6]

Enterococcus faecalis is a gram positive, facultative anaerobe mainly isolated from the root canal treatment failure cases.[3] This is the most commonest bacteria isolated from the persistent periapical lesions after endodontic therapy.Enterococcus faecalis has the ability to survive in an extreme alkaline pH and salt concentrations. It resists bile salts, detergents, heavy metals, ethanol, azide & desiccation and possess survival capability up to 60^0C.[5]None of the endodontic irrigants proved to be successful against enterococcus faecalis biofilm except Sodium hypochlorite.

Sodium hypochlorite is the most effective broad spectrum antimicrobial irrigant used widely in the endodontic treatment. The antibacterial action of sodium hypochlorite is directly proportional to its concentration.[13] Higher the concenteration of sodium hypochlorite, greater the antibacterial activity. 5.25% concenteration of sodium hypochlorite alone has the ability of biofilm disruption. But the major drawback associated with higher concenteration of sodium hypochlorite is its periapical toxicity. Moreover, it has adverse effects on mechanical properties of dentin such as alteration in modulus of elasticity and flexural strength leading to fracture of the teeth. Another major disadvantage of sodium hypochlorite is its deleterious effect on dentin bonding.[56]

To overcome the drawbacks associated with higher concenterations of sodium hypochlorite, various studies have been done regarding the antibacterial activity of lower concentration of sodium hypochlorite. But the lower concentrations of sodium hypochlorite lacks the biofilm disrupting ability.[57]Several studies have shown that the antimicrobial activity of lower

concentration of sodium hypochlorite could be increased by warming ,agitation and addition of other agents.[58]

Chlorhexidine is widely used as an endodontic irrigant and as a final rinse in irrigation regimen. The major advantages of chlorhexidine are its substantivity, biocompatability and MMP inhibition in bonding procedures.Regarding antimicrobial activity, Chlorhexidine doesn't possess the biofilm destruction potential.[59]

To counteract the disadvantages of sodium hypochlorite and chlorhexidine, Niaziet al evaluated antibacterial efficiency of low concentration of 1% sodium hypochlorite and 0.2% chlorhexidine combined with proteolytic enzymes. It was concluded that chlorhexidine when combined with proteolytic enzymes showed synergistic antimicrobial effect.[16,60]

Trypsin is a proteolytic enzymes or serine protease . This proteolytic enzyme has the action on protein and splits in to protein fragments. [15]

Hence the present study aimed to evaluate the antienterococcus faecalis activity of various concentrations of sodium hypochlorite and 2% chlorhexidine combined with trypsin under confocal laser scanning microscope.

Confocal laser scanning microscope (CLSM) is considered to be efficient tool for quantification of viability cells than the traditional culture techniques.[61] Traditional colony count can only detect bacteria that possess the ability to initiate cellular division. Moreover, the bacteria can be sensitive to temperature, media and duration of incubation. Time required for response values from 24 hours to more than a week.[62]CLSM is an optical microscope which uses laser light and electronic system for image processing . The main principle used for image acquisition is the laser light from light source focused on the sample with respective dye applied and the resulting fluorescence from the sample was captured by the

objective camera.The difference in colour of fluorescence(red indicates dead cells or green indicates live cells) enables to analyse the viability of biofilm containing microbial cells.[63]

In the present study, there is no significant difference among Group1,2,3,4(5.25% NaOCl, 3% Naocl+trypsin, 1%Naocl+trypsin & 2% chlorhexidine+trypsin respectively). The results showed that the antimicrobial activity of lower concentration of sodium hypochlorite(3% &1%) and 2% chlorhexidine was greatly enhanced by the addition of trypsin. This might be due to the hydrolytic action of trypsin on peptide bonds involving lysine and arginine. Due to this hydrolytic action , the protein fragments in biofilm matrix gets disrupted and exposed the microbial cells.[16]Trypsin also acts oncell surface proteins of microbial cells which are used to coaggregate the cells in biofilm. Denaturing of surface proteins leads to protein fragmentation and in turn leads to loss of coaggregation of microbial cells.[16]

In the present study, ultrasonic activation played a major role in improving the synergistic effect of trypsin. Enhanced antimicrobial activity of lower concentrations of sodium hypochlorite and 2% chlorhexidine might also be due to the ultrasonic agitation done in this study.

Several studies showed that the ultrasonic activation plays an important role in biofilm disrupting ability of trypsin. Furtherthe antimicrobial action of sodium hypochlorite is enhanced by the ultrasonic agitation compared to conventional irrigation technique.[64] In the present study all the experimental groups showed excellent antimicrobial effect except saline group. The synergistic effect of trypsin demonstrated in the present study might be due to the ultrasonic activation of irrigants. In a study done by Niazi et al, it was established that trypsin with ultrasonic agitation exhibited efficient biofilm disruption and antimicrobial activity.[16]The mechanism behind the action of ultrasonic can be attributed to acoustic

cavitation and heatgeneration. The mechanical agitation leads to the disruption of biofilm matrix. The heat generation during ultrasonic activation also enhances the antimicrobial effect of experimental irrigants.[64]Several studies showed that an increase in temperature of sodium hypochlorite exhibited enhanced antibacterial activity when compared to irrigants at normal room temperature. [24]

Results obtained in the present study might be due to the combined action of enzymic solution, antimicrobial solution, and ultrasonic activation (mechanical agitation and heat energy). Biofilm disruption by enzymic action (trypsin,) and mechanical agitation (ultrasonics) had exposed the microbial cells to antimicrobial irrigants (Naocl and CHX).[16]

Trypsin, a proteolyticenzyme showed a positive effect on antiinflammatory property and wound healing process.[65]Trypsin also showed some limited irritating effect on respiratory mucous membranes.[16]Hence further studies are required to explain about the biocompatibility and wound healing effect of trypsin over the periapical tissues.

SUMMARY

Seventy five single rooted teeth were selected and the dentin specimens were prepared from the middle third of the root. Dentin specimens were immersed in peptone agar medium eppendorf tubes. Enterococcus faecalis strains were inoculated and cultured for 21 days at 37^0c. Then the dentin specimens were exposed to the experimental irrigants (5.25% Naocl, 3% Naocl + trypsin, 1%Naocl+trypsin, 2% chlorhexidine + trypsin, normal saline) and evaluated under confocal laser scanning microscope to determine the antibacterial efficacy of experimental irrigants.

The findings of the present study were:

There was no statistically significant difference found among the groups 1,2,3,4. Group5(negative control) showed statistically significant difference compared to groups 1,2,3,4.

CONCLUSION

There was no significant difference among the groups regarding antienterococcus faecalis activity except negative control.

Based on the results of the present study, it can be concluded that the irrigation protocol with 1% trypsin followed by lower concentrations of sodium hypochlorite(1% &3%) and 1 % trypsin followed by 2% chlorhexidine showed equivalent antibacterial effect compared to 5.25% sodium hypochlorite. Further studies are needed to evaluate the effect of trypsin over the physical properties of root canal dentin.

References

1. Bystrom A, Sundqvist G Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. Scand J Dent Res. 1981:89(4):321-8.
2. Sundqvist G, Figdor D, Persson S, Sjogren U. Microbiologic Analysis Of Teeth With Failed Endodontic Treatment And The Outcome Of Conservative Re-Treatment. Oral Surg Oral Med Oral Pathol Oral RadiolEndod 1998; 85(1):86 –93.
3. Rôças IN1, Siqueira JF Jr, Santos KR.Association of Enterococcus faecalis with different forms of periradicular diseases. J Endodon. 2004; 30(5): 315 -320.
4. Morgental RD, Singh A, Sappal H, Kopper PM, Vier‐Pelisser FV, Peters OA. Dentin inhibits the antibacterial effect of new and conventional endodontic irrigants. J Endod 2013;39(3):406‐10.
5. Stuart CH1, Schwartz SA, Beeson TJ, Owatz CB. Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. J Endod. 2006; 32(2):93-8.
6. NeelakantanP,RomeroM,VeraJ ,Daood U, Khan A.U,Yan A, CheungG.S.P. Biofilms in endodontics—Current status and future directions. Int. J. Mol. Sci. 2017, 18(8):E1748.
7. Kapil Jhajharia, Abhishek Parolia,K Vikram Shetty and Lata Kiran Mehta· Biofilm in endodontics: a review. J IntSoc PrevCommunity Dent. 2015; 5(1): 1–12.
8. Shehab El-DinMohamed Saberand Soha A. El-Hady. Development of an intracanal mature *Enterococcus faecalis* biofilm and its susceptibility to some antimicrobial intracanal medications; an *in vitro* study.Eur J Dent. 2012; 6(1): 43–50.

9. Mohammadi Z. Sodium hypochlorite in endodontics: an update review. Int Dent J 2008; 58(6): 329–341.
10. Clarkson RM, Moule AJ, Podlich H Kellaway R, Macfarlane R, Lewis D, Rowell J. Dissolution of porcine incisor pulps in sodium hypochlorite solutions of varying compositions and concentrations. Aust Dent J 2006 51(3): 245-251.
11. Stojicic S, Zivkovic S, Qian W, Zhang H, Haapasalo M. .Tissue dissolution by sodium hypochlorite: Effect of concentration, temperature, agitation, and surfactant.J Endod. 2010;36(9):1558-62
12. Hauman CH, Love RM. Biocompatibility of dental materials used in contemporary endodontic therapy: a review. Part 1. Intracanal drugs and substances. Int Endod J 2003 36(2): 75-85.
13. ForoughReyhaniM, RezagholizadehY, NarimaniMR, RezagholizadehL, MazaniM, Barh aghiMHS , Mahmoodzadeh Y. Antibacterial effect of different concentrations of sodium hypochlorite on Enterococcus faecalis biofilms in root canals. J Dent Res Dent Clin Dent Prospects. 2017;11(4):215-221.
14. Zehnder M. Root canal irrigants. J Endod 2006;32(5):389-98.
15. Mukherji R, Patil A, PrabhuneA . Role of Extracellular Proteases in Biofilm Disruption of Gram Positive Bacteria with Special Emphasis on *Staphylococcus aureus Biofilms*. EnzEng2015;4(1):126.
16. Niazi SA, Clark D, Do T, Gilbert SC, Foschi F, Mannocci F, Beighton D The effectiveness of enzymic irrigation in removing a nutrient stressed endodontic multi-species biofilm. International Endodontic Journal 2014 ;47(8):756-68.

17. Siqueira JF, Jr, Batista MM, Fraga RC, de Uzeda M. Antibacterial effects of endodontic irrigants on black-pigmented Gram-negative anaerobes and facultative bacteria. J Endod. 1998;24(6):414–6.
18. Heling I, Chandler NP. Antimicrobial effect of irrigant combinations within dentinal tubules. Int Endod J 1998;31(1):8-14.
19. SprattDA, PrattenJ, WilsonM,GulabivalaK. An invitro evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. Int Endod J 2001;34(4):300–7.
20. Heling I, Rotstein I, Dinur T, Szwec-levine Y, Steinberg D. Bactericidal and cytotoxic effects of sodium hypochlorite and sodium dichloroisocyanurate solutions in vitro. J Endod. 2001;27(4):278–280.
21. Sassone LM, Fidel RA, Fidel SR, Dias M, Hirata RJ. Antimicrobial activity of different concentrations of NaOCl and chlorhexidine using a contact test. Braz Dent J. 2003;14(2):99–102.
22. Önçağ Ö, Hoşgör M, Hilmioğlu S, Zekioğlu O, Eronat C, Burhanoğlu D. Comparison of antibacterial and toxic effects of various root canal irrigants. Int Endod J. 2003;36(6):423–32.
23. Ercan E, Ozekinci T, Atakul F, Gül K. Antibacterial activity of 2% chlorhexidine gluconate and 5.25% sodium hypochlorite in infected root canal: in vivo study. J Endod. 2004. February;30(2):84–7.
24. Sirtes G, Waltimo T, Schaetzle M, Zehnder M. The effects of temperature on sodium hypochlorite short-term stability, pulp dissolution capacity, and antimicrobial efficacy. J Endod 2005;31(9):669-71.

25. Berber VB, Gomes BP, Sena NT, Vianna ME, Ferraz CC, Zaia AA, Filho Fj..Efficacy of various concentrations of NaOCl and instrumentation techniques in reducing *Enterococcus faecalis* within root canals and dentinal tubules. Int Endod J 2006;39(1):10-7.
26. Khademi AA, Mohammadi Z, Havaee A Evaluation of the antibacterial substantivity of several intra-canal agents. Australian Endodontic Journal 2006,32(3), 112–115.
27. Dunavant TR, Regan JD, Glickman GN, Solomon ES, Honeyman AL Comparative evaluation of endodontic irrigants against Enterococcus faecalis biofilms. Journal of Endodontics 2006,32(6): 527–31.
28. Oliveira DP, Barbizam JV, Trope M, Texeira FB. In vitro antibacterial efficacy of endodontic irrigation against Enterococcus faecalis. Oral Surg Oral Med Oral Path Oral RadiolEndod. 2007;103(5):702–6.
29. MontseMercade, Fernando ,Duran-Sindreu, Sergio kutler,and MiguelRoig, NurieDurany. Antimicrobial efficacy of 4.2% sodium hypochlorite adjusted to oH 12,7.5 &6.5 in infected root canals.Oral Surg Oral Med Oral Pathol Oral Radiol Endod.2009: 107:295-298.
30. Arias-Moliz MT, Ferrer-Luque CM, Espigares-Garcia M, Baca P. Enterococcus faecalis biofilms eradication by root canal irrigants. J Endod. 2009;35(5):711–714.
31. Tirali RE, Turan Y, Akal N, Karahan ZC. In vitro antimicrobial activity of several concentrations of NaOCl and Octenisept in elimination of endodontic pathogens.OralSurg Oral Med Oral Pathol Oral RadiolEndod. 2009;108(5):e117–20.

32. Retamozo B, Shabahang S, Johnson N, Aprecio RM, Torabinejad M. Minimum contact time and concentration of sodium hypochlorite required to eliminate *Enterococcus faecalis*. J Endod. 2010;36(3):520–3.
33. Bhuva B[1], Patel S, Wilson R, Niazi S, Beighton D, MannocciF.The effectiveness of passive ultrasonic irrigation on intraradicular Enterococcus faecalis biofilms in extracted single-rooted human teeth. Int Endod J. 2010;43(3):241–250.
34. Shen Y, Stojicic S, Qian W, Olsen I, Haapasalo M. The synergistic antimicrobial effect by mechanical agitation and two chlorhexidine preparations on biofilm bacteria. J Endod. 2010;36(1):100– 104.
35. OzdemirHO, BuzogluHD CaltS, StabholzA, Steinberg DEffect of ethylenediamine tetra acetic acid and sodium hypochlorite irrigation on Enterococcus faecalis biofilm colonization in young and old humanrootcanaldentin: in vitro study. J Endod 201036(5):842-846.
36. Rôças IN, Siqueira JF., Jr Comparison of the *in vivo* antimicrobial effectiveness of sodium hypochlorite and chlorhexidine used as root canal irrigants: A molecular microbiology study. J Endod. 2011;37(2):143–50.
37. Ordinola-Zapata R, Bramante CM, Aprecio RM, Handysides R, Jaramillo DE. Biofilm removal by 6% sodium hypochlorite activated by different irrigation techniques. Int Endod J 2014;47(7):659–666.
38. Morgental RD, Singh A, Sappal H, Kopper PM, Vier-Pelisser FV, Peters OA, et al. Dentin inhibits the antibacterial effect of new and conventional endodontic irrigants. J Endod 2013;39(3):406 -10.

39. Neelakantan P, Cheng CQ, Mohanraj R, Sriraman P, Subbarao C, Sharma S Antibiofilm activity of three irrigation protocols activated by ultrasonic, diode laser or Er:YAG laser in vitro. Int Endod J 2015, 48(6):602–610.
40. Gonçalves, Lucio & Renan Oliveira Brito, Julia & Cezar Machado Oliveira, Julio. Effectiveness of chlorhexidine and sodium hypochlorite to reduce Enterococcus faecalis biofilm biomass. Journal of Dentistry and Oral Hygiene2014;6: 64-69.
41. Wong DT, Cheung GS. Extension of bactericidal effect of sodium hypochlorite into dentinal tubules. J Endod. 2014;40(6):825–829.
42. De Almeida AP, Souza MA, Miyagaki DC, Dal Bello Y, Cecchin D, Farina AP. Comparative evaluation of calcium hypochlorite and sodium hypochlorite associated with passive ultrasonic irrigation on antimicrobial activity of a root canal system infected with *Enterococcus faecalis*: an *in vitro* study. J Endod. 2014;40(12):1953–1957.
43. Rita Noites, Cidália Pina-Vaz, Rita Rocha, Manuel Fontes Carvalho, Acácio Gonçalves, and Irene Pina-vaz,. Synergistic antimicrobial action of chlorhexidine and ozone in endodontic treatment. Biomed Res Int. 2014:Article ID592423.
44. Du T, Wang Z, Shen Y, Ma J, Cao Y, Haapasalo M. Effect of long-term exposure to endodontic disinfecting solutions on young and old *Enterococcus faecalis* biofilms in dentin canals. J Endod. 2014;40(4):509–514.
45. Salian Shailaja, Sham S Bhat, Sundeep Hegde K.Comparison between the Antibacterial Efficacies of Three Root Canal Irrigating Solutions: Antibiotic Containing Irrigant, Chlorhexidine and Chlorhexidine + Cetrimide.. Oral health dentmanag.2013; 12:295-299.

46. Ruqshun Anjum MG, SujathaI, Sharath, Chandra SM. Antimicrobial efficacy of various irrigating solutions on enterococcus faecalis in root canals: An invitro study. Int. J. Appl.Dent sci 2015;1(4),94-97.

47. Ruiz-Linares M, Aguado-Pérez B, Baca P, Arias-Moliz MT, Ferrer-LuqueCM. . Efficacy of antimicrobial solutions against polymicrobial root canal biofilm. Int Endod J 2017;50(1):77–83.

48. SevinçAktemurTürker,MehtapHülya Aslan,Sevilay Karahan,EmelUzunoğlu. Effect of Temperature , Concentration , and Contact Time of Sodium Hypochlorite on Enterococcus Faecalis." Clinical dentistry and research2015 39(2):69_73.

49. Giardino L, Estrela C, Mohammadi Z, Palazzi F. Antibacterial power of sodium hypochlorite combined with surfactants and acetic acid. Braz Dent J. 2014;25(4):289–94.

50. Yang Y., Shen Y., Wang Z., Huang X., Maezono H., Ma J., Cao Y., Haapasalo M. Evaluation of the Susceptibility of Multispecies Biofilms in Dentinal Tubules to Disinfecting Solutions. J. Endod. 2016;42(8):1246–1250.

51. Pladisai P, Ampornaramveth RS, Chivatxaranukul P. Effectiveness of Different Disinfection Protocols on the Reduction of Bacteria in Enterococcus faecalis Biofilm in Teeth with Large Root Canals. J Endod. 2016; 42(3): 460–464.

52. Turki, Alaa. Efficacy of sodium hypochlorite 5.2% on enterococcus faecalisAND it's diagnosis with antibiogram ISOLATED from infected pulp.ejpmr, 2016, 3(8), 67_72.

53. MiçooğullarKurtS&Çalışkan MK. Efficacy of chlorhexidine as a final irrigant in one-visit root canal treatment: A prospective comparative study. International Endodontic Journal. 2018, 51(10):1069-1076.

54. MohmmedSA ,ViannaME,Penny M,HiltonSR,Mordan NJ, KnowlesJC. Investigations into in situ Enterococcus faecalis biofilm removal by passive and active sodium hypochlorite irrigation delivered into the lateral canal of a simulated root canal model ,2017, International Endodontic Journal,JEndod2018;51(6), 649–662.
55. Yoo A, Rossi-Fedele G, Kidd SP, Rogers AH, Zilm PS. Association between Extracellular Material and Biofilm Formation in Response to Sodium Hypochlorite by Clinical Isolates of Enterococcus faecalis 2017, 44(2):269-273.
56. ZahedMohammadi,ShapourYaripour, SousanShalavi, FlavioPalazzi,andSaeed Asgarya.Root Canal Irrigants and Dentin Bonding: An Update.Iran Endod J. 2017 ; 12(2): 131–136.
57. Clegg MS, Vertucci FJ, Walker C, Belanger M, Britto LR. The effect of exposure to irrigant solutions on apical dentin biofilms in vitro. J. Endod. 2006;32(5):434–437.
58. TopbasC., & Adiguzel O. Endodontic Irrigation Solutions: A Review. *International Dental Research*, 2017,*7*(3), 54-61.
59. Mohammadi Z[1], Abbott PV.The properties and applications of chlorhexidine in endodontics.Int Endod J. 2009 ;42(4):288-302
60. NiaziSA, Al-Ali WM, Patel S, FoschiF, MannocciF.Synergistic effect of 2% chlorhexidine combined with proteolytic enzymes on biofilm disruption and killing.Int Endod J. 2015;48(12):1157-67.
61. Wong DT, Cheung GS. Extension of bactericidal effect of sodium hypochlorite into dentinal tubules.J Endod. 2014;40(6):825-9.

62. Shen Y, Stojicic S, Haapasalo M. Bacterial viability in starved and revitalized biofilms: comparison of viability staining and direct culture. J Endod 2010;36(11):1820-3.
63. Paddock SW Principles and practices of laser scanning confocal microscopy.Mol Biotechnol. 2000;16(2):127-49.
64. PlotinoG, Pameijer CH, Grande NM, Somma F. Ultrasonics in endodontics: a review of literature. J Endod 2007;33(2):81-95.
65. White MJ, Glenn M, Gomer RH . Trypsin potentiates human fibrocyte differentiation. PLoS One 2013;7, 8(8): e70795.

APPENDIX I

INSTITUTIONAL ETHICAL COMMITTEE

KSR INSTITUTE OF DENTAL SCIENCE & RESEARCH

KSR Kalvi Nagar, Tiruchengode-637 215, Tamilnadu.
Phone : 04288-274981, Fax : 04288-274761,
email : ksrdentalcollege@yahoo.com

Chairman
Dr. PHILIP ROBINSON, Ph.D
Prof. & Head Dept. of Biotechnology
KSR College of Technology,
KSR Kalvi Nagar, Tiruchengode.

Member Secretary
Dr. G.S. KUMAR, MDS.,
Principal,
KSR Institute of Dental Science & Research,
KSR Kalvi Nagar, Tiruchengode.

Members

Dr.G.Ayppadasan,Ph.D.,
Biotechnologist

Mr.A.Thirumoorthi,M.A.B.L.,
Human Activist

Dr.R.Renuka,M.D.S.,(Perio),M.Sc.,
Family Counsellor

Dr.M.Rajmohan,MDS,(Oral Path)

Dr.R.Prakash, MDS,(PHD)

Dr.Suman, M.D.S.,(OMDR)

Dr.Sharath Ashokan,MDS.,(Pedo)

Dr.G.Rajeswari,Ph.D.,(Biochemistry)

Dr.K.Karthick,MDS.,(Cons.Dent.)

Mr.V.Mohan,M.Sc.,M.Phil.,(Physicist)

Mr.A.P.S.Raja,B.A.,
(Layperson)

Ref.: 147/KSRIDSR/EC/2016 **Date : 19.12.2016**

To

Dr.S.Elangovan,
Postgraduate Student,
Dept. of Conservative Dentistry & Endodontics,
KSR Institute of Dental Science & Research,

*****

Your dissertational study titled "ANTIBACTERIAL EFFECT OF VARIOUS CONCENTRATIONS OF SODIUM HYPOCHLORITE AND 2% CHLORHEXIDINE COMBINED WITH PROTEOLYTIC ENZYME AGAINST ENTEROCOCCUS FAECALIS BIOFILM – AN INVITRO STUDY" presented before the ethical committee on 16th Dec. 2016 has been discussed by the committee members and has been approved.

You are requested to adhere to the ICMR guidelines on Biomedical Research and follow good clinical practice. You are requested to inform the progress of work from time to time and submit a final report on the completion of study.

Signature of Member Secretary
(Dr.G.S.Kumar)

APPENDIX II

URKUND

Urkund Analysis Result

Analysed Document:	FLANGO THESIS plagiarism.docx (D47679573)
Submitted:	2/7/2019 9:19:00 AM
Submitted By:	eladentsurge@gmail.com
Significance:	3 %

Sources included in the report:

varshini.pdf (D34306577)
My Dissertation Final .docx (D45631487)
Vincy Final Thesis.pdf (D34366018)
https://www.science.gov/topicpages/c/chlorhexidine+gluconate+chx
https://worldwidescience.org/topicpages/s/sodium+hypochlorite+effect.html
https://www.science.gov/topicpages/s/sodium+hypochlorite+concentration.html
https://www.science.gov/topicpages/e/enterococcus+faecalis+fk+23

Instances where selected sources appear:

9

APPENDIX III

This is to certify that this dissertation work titled "**ANTIBACTERIAL EFFECT OF VARIOUS CONCENTRATIONS OF SODIUM HYPOCHLORITE AND 2% CHLORHEXIDINE COMBINED WITH PROTEOLYTIC ENZYME AGAINST ENTEROCOCCUS FAECALIS BIOFILM – AN INVITRO STUDY**" of the candidate **Dr.S.ELANGOVAN** with registration number **241617401** for the award of "**MASTER OF DENTAL SURGERY**" in the branch of **CONSERVATIVE DENTISTRY AND ENDODONTICS**. I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **3%** percentage of plagiarism in the dissertation.

09/02/2019

Guide & Supervisor sign with s

PROFESSOR & HOD,
DEPT OF CONSERVATIVE
DENTISTRY & ENDODONTICS,
K. S. R. Institute of Dental
Science & Research,
K. S. R. KalviNagar,
Thiruchengode, Tamil Nadu.